Volume 49 OCTOBER, 1951 Number 4

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VOLUME 49 OCTOBER, 1951 NUMBER 4

BIOASSAY OF ESTROGENS USING THE MOUSE UTERINE RESPONSE[1]

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THIS report is concerned with an analysis of an estrogen bioassay method employing the immature mouse uterus which is sensitive, precise, convenient, and inexpensive. The data include studies with pure compounds as well as application of the method to the assay of urinary extracts.

ANIMALS, MATERIALS, AND METHOD

All Swiss albino mice were obtained from Albino Farms. The mice were 23 to 25 days of age at the start of the assay. They were injected subcutaneously once daily for three days with a corn oil solution of the hormone. The daily dose was contained in 0.1 cc. of oil. Twenty-four hours after the last injection the animals were sacrificed and the uterine and body weights were determined. The uterine ratio was calculated as one hundred times the uterine weight in milligrams divided by the body weight in grams.

The experimental design of Bliss (1944) was employed. Four equal-sized groups of animals were injected, two with a standard preparation, and two with an unknown. The concentrations of standard and unknown were chosen so that the following relationship holds:

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}.$$

With this experimental design and the formulations of Bliss (1944), it is possible to calculate: (1) the relative potency of an unknown preparation, (2) the standard error of the potency ratio, (3) the mean slope (b), (4) the significance of the difference between the respective slopes (t), and (5) the index of precision (λ). The smaller the value of λ, the more precise the assay. Most of the calculations were based on uterine ratio

Received for publication September 25, 1950.

[1] Supported in part by a grant (RG 1050) from the Division of Grants and Fellowships, National Institute of Health, U. S. Public Health Service.

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versus logarithm of the dose, but for estrone there have been included calculations using the relationship of logarithm of uterine ratio versus logarithm of dose. This is necessary when the range of dosage is wide (0.04 to 0.32 microgram). Under these conditions it was found that a log dose-log response relationship resulted in a straight line whereas the log dose-response relationship did not.

For the preparation of urinary extracts, the freshly collected urine was acidified with concentrated hydrochloric acid (15 cc. of acid per 100 cc. of urine), hydrolyzed under the reflux for fifteen minutes, and cooled under the tap. Sodium chloride (10 grams per 100 cc.) was added to the acidified hydrolyzed urine which was then extracted with one-fourth volumes of benzene three times. The combined benzene extracts were evaporated to dryness *in vacuo* and dissolved in ether. The ether fraction was extracted with saturated sodium bicarbonate to remove the organic acids and then with 2.5 N sodium hydroxide to remove the phenols. The aqueous sodium hydroxide fraction containing the estrogens was acidified with sulfuric acid and reextracted with ether. The ether solution, after washing with water, was evaporated to dryness and the resulting residue constituted the total estrogenic fraction on which the bioassays were performed.

RESULTS

Pure Compounds

Table 1 lists the mean body weights ±S.E. and the mean uterine ratios ±S.E. obtained with various doses of estrogens by two different investigators in this laboratory. From this table, it can be seen that the sensitivity of the uterus to the three estrogens varies: 0.02–0.06 microgram being a useful assay range for α-estradiol, 0.04–0.32 microgram being a

TABLE 1. RESPONSE OF MOUSE UTERUS TO ESTROGENS

Estrogen	Total dose μg.	No. of mice	Mean body weight gm. ±S.E.	Mean uterine ratio ±S.E.	Investigator No.
0	0	30	9.6±0.3	111± 4	1
Estrone	0.04	52	9.5±0.2	140± 5	1
	0.08	38	9.6±0.3	162± 7	1
	0.16	54	9.9±0.2	285±11	1
	0.32	37	10.1±0.2	431±17	1
0	0	57	11.2±0.3	103± 4	2
Estrone	0.10	30	12.3±0.4	186± 7	2
	0.20	21	11.3±0.5	288±16	2
	0.30	21	11.7±0.6	414±23	2
Estriol	1.00	10	12.5±0.4	149± 5	2
	2.00	11	12.0±0.9	163± 7	2
	3.00	24	9.5±0.4	168± 6	2
	4.00	10	10.2±0.9	190±12	2
	8.00	10	10.6±0.5	246±12	2
	9.00	26	10.3±0.3	228± 8	2
	16.00	9	10.0±0.8	297±16	2
Estradiol	0.02	11	11.0±0.4	231±14	2
	0.04	11	11.5±0.4	302±18	2
	0.06	11	11.0±0.4	362±22	2

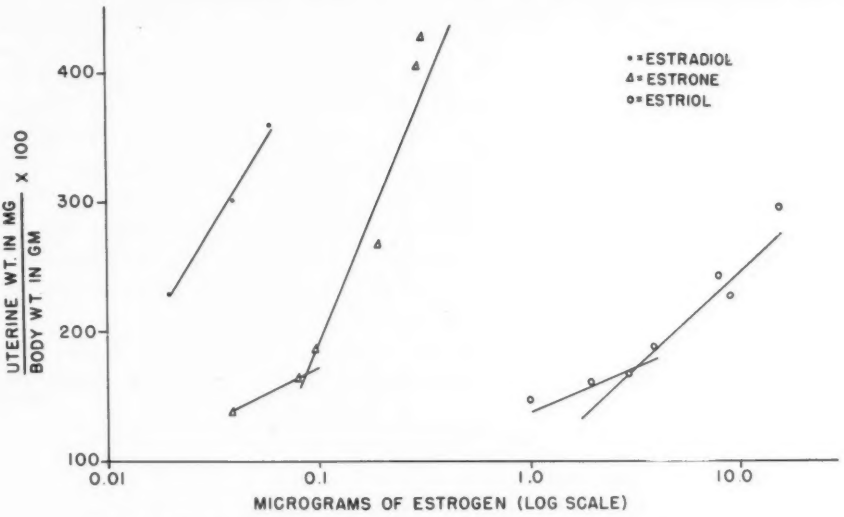

FIG. 1. Log dose—Response Relationships Between Uterine Ratio and Estrone, Estradiol, and Estriol.

useful assay range for estrone, and 1–16 micrograms being required to assay for estriol. Figure 1 illustrates the log dose-response relationship.

Table 2 records the regression coefficients for estrone, α-estradiol and estriol calculated by the method of Fisher (1934), using the log dose-response relationship. The slope for estrone in the range 0.04–0.08 microgram is 50.5. In the range from 0.1–0.32 microgram the slope for estrone is 442. For estriol, the slope in the range 1–4 micrograms is 50.4, and in the range 2–16 micrograms it is 149. The response to estradiol, in the range 0.02–0.06 microgram, has a slope of 270, which is not significantly different ($t=0.029$) from the slope for estrone of 442. However, the respective constants are significantly different ($t=2.56$) indicating a real

TABLE 2. COMPARISON OF MOUSE UTERINE RESPONSE TO ESTRONE, ESTRADIOL AND ESTRIOL

Relationship: Log dose—Response

Estrogen	Total no. mice	Dosage levels μg.	Regression coefficients (Fisher method)
Estrone	90	0.04, 0.08	$y=213+50.5 \log x$
Estrone	81	0.1, 0.3	$y=619+440 \log x$
Estrone	33	0.1, 0.2, 0.3	$y=609+444 \log x$
Estradiol	33	0.02, 0.04, 0.06	$y=687+270 \log x$
Estriol	55	1.0, 2.0, 3.0, 4.0	$y=149+50.4 \log x$
Estriol	90	2.0, 3.0, 4.0, 8.0, 9.0, 16.0	$y=99+149 \log x$

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difference in potencies. The potency of α-estradiol relative to estrone as calculated by the Fisher method is 5.41. At low response levels, over the narrow range for estrone of 0.04–0.08 microgram and for estriol of 1.0–4.0 micrograms, the slopes of the log dose-response relationships are identical ($b = 50.5$) for the two estrogens. The potency of estriol relative to estrone in that range is 0.05. At higher response levels, over the more extended ranges of 0.1–0.32 microgram for estrone and 2–16 micrograms for estriol, the slopes (442 and 149, respectively) do differ significantly. Therefore, at other than low levels of response, estriol must be assayed against an estriol standard, while α-estradiol and estrone can be assayed against each other by using the proper conversion factors.

In order to test the utility of the method, estrone and estriol were assayed against themselves. In Tables 3, 4 and 5 are listed the results of

TABLE 3. THE ASSAY OF ESTRONE USING THE MOUSE UTERINE RESPONSE AND A SIMPLIFIED DESIGN

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}$$

(Unknown = Standard)

Relationship: log dose-log response

N	Dosage levels μg.	b	S	λ	t	Potency ratio ± S.E. %
18	0.32;0.08	0.670	0.131	0.195	0.901	102 ± 11
18	0.32;0.16	0.547	0.106	0.194	1.890	117 ± 12
18	0.32;0.16	0.531	0.121	0.230	1.438	97 ± 12
18	0.16;0.08	0.797	0.114	0.143	0.260	105 ± 8
18	0.08;0.04	0.351	0.093	0.264	0.860	100 ± 14

the Bliss (1944) calculations applied to estrone and estriol using various numbers of animals, various ratios of high to low dose, and assaying on various portions of log dose-response and log dose-log response curves. No consistent pattern appears of desirability of using one or another ratio of high to low dose, as indicated by values of λ. In general, it is better not to assay too near the minimum or maximum response levels.

In Table 3, eighteen animals were used at each dose level of each estrone preparation. The slope, index of precision (λ), t values and potency ratios are calculated on the basis of a log dose-log response relationship. The average slope is 0.539, the average λ is 0.205 ± 0.020, and the average potency ratio (theoretically 100 per cent since unknown and standard were the same) is 104 ± 11 per cent. In all cases the slopes of unknown and standard were not significantly different (all t values were less than 2.09).

In Table 4, the Bliss calculations are based on a log dose-response rela-

tionship and ten animals were used in each group. The mean slope was 440 ± 46 and the mean value for λ was 0.176 ± 0.021. The potency ratio varied from 89 ± 11 per cent to 109 ± 15 per cent. The mean potency ratio was 99 ± 13 per cent.

TABLE 4. THE ASSAY OF ESTRONE USING THE MOUSE UTERINE RESPONSE AND A SIMPLIFIED DESIGN

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}$$

(Unknown = Standard)

Relationship: log dose-response

N	Dosage levels μg.	*b*	*S*	λ	*t*	Potency ratio ±S.E. %
10	0.16;0.08	232.7	70.0	0.301	0.282	104 ± 26
10	0.16;0.08	361.0	43.5	0.1205	0.432	95 ± 8
10	0.32;0.08	447.5	74.3	0.166	0.531	89 ± 11
10	0.32;0.08	465.0	95.1	0.204	0.1675	96 ± 14
10	0.32;0.16	648.0	87.7	0.135	0.689	106 ± 10
10	0.32;0.16	552.0	93.0	0.168	0.0221	94 ± 12
10	0.20;0.10	336.0	63.0	0.1875	0.216	109 ± 15
10	0.30;0.10	474.0	60.5	0.1275	1.72	97 ± 9

In Table 5 are listed the Bliss calculations for estriol, using four or five animals per group, and, in one case, ten animals per group. Using four or five animals per group, the mean slope was 162 ± 17, indicating a flatter curve than for estrone. The value for λ was higher (0.290 ± 0.040) and the standard error of the potency ratio greater (126 ± 43 per cent). However, in the one assay where ten animals were used in each group, the value of λ was 0.1825, and the potency ratio was 96 ± 13 per cent, indicating that the assay method is usable even though the curve is flatter.

TABLE 5. THE ASSAY OF ESTRIOL USING THE MOUSE UTERINE RESPONSE AND A SIMPLIFIED DESIGN

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}$$

(Unknown) = (Standard)

Relationship: log dose-response

N	Dosage levels μg.	*b*	*S*	λ	*t*	Potency ratio ±S.E. %
5	4;2	101.0	36.6	0.362	0.329	129 ± 52
5	8;2	145.5	57.9	0.398	0.665	133 ± 66
5	8;4	191.5	45.7	0.2385	0.578	93 ± 23
4	16;4	183.0	43.9	0.240	0.0854	159 ± 46
4	16;8	187.5	39.7	0.2115	1.105	117 ± 29
10	9;3	113.3	20.7	0.1825	0.520	96 ± 13

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Urinary Extracts

Table 6 records the response of the mouse uterus to estrone and an extract prepared from normal men's urine. The study was designed to compare the responses of the two materials, particularly from the point of view of parallelism of the dose response curves. If the slopes of the respective dose response curves are not significantly different, then it is possible to express the urinary estrogen concentrations in terms of estrone. Actually the slope of estrone was found to be 251.2 ± 20.4 and that of the extract run simultaneously derived from normal men's urine was 265.8 ± 35.8. Calculation of the significancy of differences between the slopes by the method of Fisher (1934) gave a value of $t = 0.362$. This indicates that the slopes were not significantly different. Calculation of the estrogenic content of a pooled sample of men's urine indicated that one liter of urine contained the equivalent of 4.1 μg. (41 I.U.) of estrone. The error of determination at $P = 0.95$ was -20 per cent to $+26$ per cent.

TABLE 6. UTERINE RESPONSE TO ESTRONE AND EXTRACT OF MEN'S URINE
(Immature Mouse)

Estrogenic material	Total dose	No. of mice	Uterine ratio* ±S.E.
0	0	57	100 ± 4
Estrone	0.08 μg.	9	270 ± 10
	0.16 μg.	10	330 ± 6
	0.32 μg.	11	420 ± 17
Pooled men's urine	27 cc.	10	300 ± 10
	54 cc.	8	370 ± 29
	108 cc.	10	460 ± 19

* Uterine Ratio $= \dfrac{100 \times \text{Ut. Wt. (mg.)}}{\text{B. W. (g.)}}$

b (estrone) $= 251.2 \pm 20.4$
b (urine) $= 265.8 \pm 35.8$

For the assay of individual urines, the design of Bliss (1944) was used. Twenty-seven samples of men's urine were studied. Two concentrations of the standard (0.1 μg. and 0.2 μg. of estrone) were run in parallel with two concentrations of the urinary extract. In fourteen assays, the slopes of the standard and unknown were not significantly different (Table 7), while in the remaining thirteen assays significantly different slopes were found. In the bulk of the assays in which the slopes (unknown and standard) were significantly different, the concentration of the unknown was either considerably greater or smaller than that of the standard. Studies on the pure compounds had indicated that a log dose-log response relationship fitted a straight line better than the log dose-response relationship when small and

TABLE 7. ESTROGEN ASSAY OF MEN'S URINE

(Trials in which slopes of unknown and standard were not significantly different)

$$\frac{\text{High Dose Estrone}}{\text{Low Dose Estrone}} = \frac{\text{High Dose Urine Extract}}{\text{Low Dose Urine Extract}} = 2$$

Sample number	Number of mice	Slope b	Index of precision λ	Significance of difference between slopes t	Potency ratio M ± S.E.	Estrone equivalent I.U./day
1	6	584	0.111	0.308	92 ± 11	23
2	8	417	0.162	0.209	73 ± 10	18
3	8	265	0.190	1.711	55 ± 11	14
4	8	313	0.117	0.901	59 ± 7	15
5	8	295	0.200	1.009	47 ± 11	12
6	8	544	0.077	0.239	114 ± 8	29
7	8	520	0.131	1.470	89 ± 10	
8	9	524	0.165	0.174	116 ± 15	58
9	9	388	0.176	1.557	88 ± 14	22
10	10	433	0.161	0.775	83 ± 10	21
11	10	549	0.112	1.188	90 ± 8	21
12	10	533	0.186	1.346	121 ± 17	61
13	10	527	0.127	0.952	131 ± 12	33
14	10	565	0.093	1.662	87 ± 6	22

large concentrations were considered. Therefore, the thirteen assays in which significantly different slopes were found were recalculated using the former relationship. Table 8 shows the results of these calculations. Of six trials where the potency ratio of unknown to standard varied between 50

TABLE 8. ESTROGEN ASSAY OF MEN'S URINE

$$\frac{\text{High dose}}{\text{Low dose}} = 2$$

Potency ratio between 50 and 150

(log dose-log response)

N	b	λ	t	M ± S.E.	Estrone equivalent I.U./day
8	0.506	0.195	1.681	72 ± 13	18
8	0.476	0.214	1.807	91 ± 16	22
9	0.652	0.113	0.405	53 ± 6	13
9	0.554	0.119	*2.810*	102 ± 8	34
10	0.661	0.126	0.898	53 ± 6	27
10	0.468	0.130	*3.840*	134 ± 15	34
		Potency ratio <50 and >150			
8	0.436	0.196	*2.384*	37 ± 5	18
8	0.448	0.087	*3.410*	187 ± 18	47
9	0.570	0.114	*2.458*	29 ± 5	15
9	0.468	0.111	*4.385*	181 ± 19	44
10	0.499	0.154	*2.650*	11 ± 1	27
10	0.502	0.152	1.699	19 ± 3	10
10	0.553	0.124	1.182	46 ± 6	23

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and 150 per cent, only in two trials were statistically significant differences in slopes found, while five of the seven trials in which the potency ratio was either less than 50 per cent or greater than 150 per cent had significantly different slopes.

DISCUSSION AND CONCLUSIONS

The mouse uterine weight method described has the advantage over vaginal smear methods of estrogen assay in that the results are objective. Using a total of forty mice, the limits of error in assaying estrone were in the neighborhood of ± 13 per cent and λ was 0.176 ± 0.021. For estriol, smaller numbers of animals were used (16–20), and the limits of error were as high as ± 35 per cent, with a λ of 0.272 ± 0.035. When forty animals were used, the error was again ± 13 per cent and λ was 0.182. The sensitivity of the mouse uterus is such that 0.04 microgram of estrone, less than 0.02 micrograms of α-estradiol, and 2 micrograms of estriol can be detected.

Other methods described in the literature are listed in Table 9, along with the slope (b), index of precision (λ), and sensitivity, where it has been possible to calculate these quantities. For estrone, only the method of Albrieux (1941) is more sensitive (the minimum effective dose was 0.005 microgram) but it has not been evaluated statistically. Brownlee's (1938) results have a value for λ of 0.115 ± 0.013. The sensitivity is 0.4 microgram.

For α-estradiol, Lauson *et al.* (1939) and Astwood (1938), using the uterine weight response of the immature rat, have shown sensitivity similar to that found in mice in the present study. In Lauson's assay, λ was 0.108 ± 0.008. Since only one estradiol assay was done in the present study, λ is not known for estradiol by the method described.

For estriol, Lauson (1939) obtained an effect on the immature rat uterus with 0.1 microgram, as compared to 2 micrograms required to show an effect on the immature mouse uterus. Lloyd (1946) found 0.2–0.4 microgram sufficient to cause vaginal opening in the immature rat, and Evans (1941) obtained an effect on mouse uterine weight with 0.04–0.16 microgram. In none of these papers do the data lend themselves to calculations of slope or of λ. Emmens (1939) found the vaginal smear response of the ovariectomized mouse to be sensitive to 0.16 microgram given in four injections, but to require 7 micrograms if the estriol was given in two injections. Emmens also points out that estrone and estradiol may be assayed against each other, but that estriol requires its own standard.

The urinary estrogens of men's urine can be adequately evaluated by means of the method herein described. Estrone is a suitable standard since the slopes of the dose response curves of standard and unknown are most frequently not significantly different. It is suggested that in each assay the unknown at two concentrations be run in parallel with the standard at two

TABLE 9. RODENT METHODS OF ESTROGEN ASSAY

Estrogen	Animal	Response	Slope (b)± S.E.	Index of Precision (λ)± S.E.	Sensitivity	References
Estrone—(S.Q.)	Immature mouse	log uterine wt. ratio	0.579± 0.075	0.205± 0.020	0.04 μg.	This report
Estrone—(S.Q.)	Immature mouse	uterine wt. ratio	440± 46	0.176± 0.021	0.04 μg.	This report
Estrone—(S.Q.)	Immature mouse	increase uterine wt.	35.9		0.05 μg.	Evans *et al.* (1941)
Estrone—(S.Q.)	Immature rat	uterine wt. ratio	88.7± 5.8	0.149± 0.017	0.6 μg.	Dorfman (unpublished)
Estrogen—6 hr. test	Immature rat	uterine wt. increase	49.0		0.25 μg.	Astwood (1938)
Estrone—(S.Q.)	Immature rat	uterine wt.			0.2 μg.	Bulbring & Burn (1935) Emmens (1939)
Estrone—(S.Q.)	Immature rat	uterine wt.	68.7	0.1515	1.0 μg.	Lauson *et al.* (1939)
Estrone—(S.Q.)	Immature rat	time of vaginal opening			0.25–0.5 μg.	Lloyd *et al.* (1946)
Estrone—(S.Q.)	Ovariectomized mouse	vaginal smear	5.46± 0.52	0.183	0.1 μg.	Emmens (1939) & Bliss (1943)
Estrone—(S.Q.)	Ovariectomized albino mouse	vaginal smear	5.353± 0.418			Emmens (1939a)
Estrone—(S.Q.)	Ovariectomized inbred CBA mouse	vaginal smear	3.375± 0.397			Emmens (1939a)
Estrone—(S.Q.)	Ovariectomized rat	uterine weight	132.4	0.115± 0.013	0.4 μg.	Brownlee (1938) & Bliss (1943)
Estrone—(S.Q.)	Ovariectomized rat	vaginal smear	7.533± 0.488		1 μg.	Curtis *et al.* (1944)
Estrone—vaginal instillation	Ovariectomized rat	vaginal smear			0.005 μg.	Albrieux (1941)
Estrone—vaginal instillation	Ovariectomized rat	% response—vaginal smear	85.4		0.05 μg.	Krichesky & Glass (1947)
Estrone—(S.Q.)	Rabbit	uterine weight			1.0 μg.	Mc'hail (1936) & Bliss (1943)
Sodium Estrone Sulfate	Immature rat	uterine weight	110± 11	0.287± 0.046	2.5 μg.	Dorfman (unpublished)
Stilbestrol	Immature rat	vaginal introitus	6.09± 0.67	0.164	0.2 μg.	Lee *et al.* (1942) Bliss (1943)
Crude extract	Ovariectomized mice	vaginal smear		0.37		Coward & Burn (1927) Gaddum (1933)
Crude extract	Ovariectomized mice	vaginal smear		0.143		Marrian & Parkes (1929) Gaddum (1943)
α-Estradiol	Immature mouse	uterine wt. ratio	270		<0.02 μg.	This report
α-Estradiol	Immature mouse	uterine wt. increase	18.4		0.01 μg.	Evans *et al.* (1941)
α-Estradiol	Immature rat	log. uterine wt.	0.457± 0.545	0.108± 0.008	0.025 μg.	Lauson *et al.* (1939) Bliss (1943)
α-Estradiol	Immature rat	uterine weight	65.3	0.0954	0.05 μg.	Lauson *et al.* (1939) This author's calculation
α-Estradiol—6 hr. test	Immature rat	uterine weight	54.2		0.0125 μg.	Astwood (1938)
α-Estradiol	Immature rat	vaginal opening			0.5 μg.	Hartman & Littrell (1945)
α-Estradiol	Ovariectomized mice	vaginal smear	248.0		0.025 μg.	Emmens (1939)
Estradiol-benzoate	Ovariectomized rat	vaginal smear	9.612± 0.863		0.2 μg.	Curtis *et al.* (1944)
Estradiol dipropionate	192 gm. guinea pig	time of vaginal opening	−37.5		5–20 μg.	Hartman *et al.* (1946)
Estradiol dipropionate	192 gm. guinea pig	time of vaginal opening	−89.8		0.004–0.06 μg.	Hartman *et al.* (1946)
Estriol	Immature mouse	uterine wt. ratio	154± 16	0.272± 0.035	2.0 μg.	This report
Estriol	Immature mouse	uterine wt. increase	no curve between 0.04 & 0.16 μg.			Evans *et al.* (1941)
Estriol	Immature rat	uterine wt.			0.1 μg.	Lauson *et al.* (1939)
Estriol	Immature rat	vaginal opening			0.2–0.4 μg.	Lloyd *et al.* (1946)
Estriol	Ovariectomized mouse	vaginal smear	7.141		0.16 μg. 7 μg.	Emmens (1939)

Unless reference is made to Bliss, Gaddum, or Emmens calculations were done by author.

concentrations. Using the calculations described by Bliss (1944) one may calculate directly the potency ratio, the standard error of the potency ratio, and the significancy of differences between the slopes of the unknown and standard. When a total of 20 animals were used for the unknown and 20 animals were used for the standard a mean standard error of 13 per cent was found.

The data presented emphasize the advantage of having the potency of

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the unknown and standard approximately the same. As the difference between the potencies increase, the use of the log dose-log response relationship becomes advantageous.

SUMMARY

A precise, convenient, and inexpensive method has been described for the assay of three naturally occurring estrogens. It is based on the straight line relationship between the logarithm of the dose and the uterine weight response of the immature mouse. When the assay is set up according to the statistical design of Bliss (1944), using a total of 40 mice, 20 on unknown and 20 on standard, the standard error of the potency ratio determined is $\pm$ 13 per cent and λ is approximately 0.18. A wider range of estrone concentration can be used if the log dose-log response relationship is employed. Estrone and α-estradiol give parallel curves over the ranges of 0.1–0.32 microgram for estrone and 0.02–0.06 microgram for α-estradiol. The potency of α-estradiol relative to estrone is 5.41. Estriol, in the range from 1–4 micrograms, and estrone, in the range from 0.04–0.08 microgram, also give parallel curves. The potency of estriol relative to estrone at this response level is 0.05. At higher levels of response, in the range for estrone of 0.1–0.32 microgram and for estriol of 2–16 micrograms, the curves differ significantly in slope, and estriol must be assayed against an estriol standard. Amounts less than 0.02 microgram of estradiol, equal to 0.04 microgram of estrone, and to 2 micrograms of estriol can be determined by this method.

The method has been applied to urinary extracts with satisfactory results.

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METABOLISM OF THE STERIOD HORMONES, METABOLISM OF PROGESTERONE AND RELATED STEROIDS[1]

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PREVIOUS *in vivo* studies in humans have indicated that progesterone may be reduced to and excreted as pregnanediol-3(α),20(α) (Venning and Browne, 1937; Buxton and Westphal, 1938; Hamblen *et al.*, 1940; Dorfman *et al.*, 1948) and pregnanol-3(α)-one-3 (Dorfman *et al.*, 1948; Venning, 1947). We have now been able to show that allopregnanediol-3(α),20(α) is also a metabolite of progesterone. Some theoretical intermediates between progesterone and the saturated diols have also been investigated. Among these are pregnanedione-3,20 and allopregnanedione-3,20. Pregnanedione-3,20 was shown to be converted to pregnanediol-3(α),20(α) while allopregnanedione-3,20 was converted to allopregnanediol-3(α),20(α) as well as allopregnanediol-3(β),20(α). As a final part of this study Δ^5-pregenol-3(β)-one-20, which has been isolated from hog testes (Ruzicka and Prelog, 1943; Haines *et al.*, 1948) was studied in women. Previously Pearlman and Pincus (1946) reported the conversion of pregnenolone to pregnanediol-3(α),20(α) in a man and in a rabbit. In our present study we have confirmed this conversion in addition to showing that pregnenolone may also be reduced to Δ^5-pregnenediol-3(β),20(α), a steroid thus far only found in the urine of patients suffering with adrenal tumors and in the urine of pregnant mares.

EXPERIMENTAL

PROGESTERONE

Four women patients with rheumatoid arthritis, ranging in age from 19 to 59 years of age, served as subjects. Progesterone[2] in sesame oil was

Received for publication October 4, 1950.

[1] Supported in part by a research grant (RG 1050) from the Division of Research Grants and Fellowships, National Institutes of Health, and the Beaumont Fund.

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[2] Generous supplies of progesterone were made available to us by the Schering Corporation.

injected intramuscularly in daily doses varying from 100 to 500 mg. The mean daily dose was 208.4 mg. Urine representing 116.4 days of treatment and 24.26 g. of progesterone was collected.

The daily urine samples were extracted with butanol by the procedure of Venning (1937). The dried residues obtained from the butanol extracts were hydrolyzed and extracted by refluxing for 3 hours in a solution of 2.1 water, 0.2 l. ethanol, 0.4 l. concentrated hydrochloric acid, and 2 l. benzene. The benzene extractions were repeated four times, the extracts collected, and taken to dryness. The total neutral fraction was prepared. Separation of the neutral fraction with Girard's reagent *T* yielded 1.4 g. in the ketonic fraction and 8.6 g. in the non-ketonic fraction.

Ketonic Fraction

The ketonic fraction was chromatographed on a column containing 30 g. aluminum oxide (Brockmann) and eluted with large volumes of carbon tetrachloride, followed by carbon tetrachloride containing 0.1% ethanol, carbon tetrachloride containing 0.2% ethanol, and carbon tetrachloride containing 0.3% ethanol. The separate fractions were then rechromatographed.

The first fraction eluted with carbon tetrachloride (310 mg.) was placed on a column containing 9 g. of a magnesium silicate-Celite (1:1) mixture. Elution with benzene yielded 120 mg. of crystals with a melting point[3] of 120° and 128–130°. A mixture of the compound with progesterone (m.p. 120° and 128–130°) melted at 120° and 128–130°.

The fractions eluted with carbon tetrachloride, 0.1% ethanol in carbon tetrachloride, and 0.2% ethanol in carbon tetrachloride, in the original chromatogram (500 mg.) were placed on a column containing 15 g. of a magnesium silicate-Celite (1:1) mixture. Elution with benzene yielded 150 mg. of crystals with a melting point of 145–149°. The acetate was prepared by heating the free compound in equal amounts of pyridine and acetic anhydride for one hour on the steam bath. The mixture yielded an acetate with a melting point of 95–98°. A mixture of the free compound with pregnanol-3(α), one-20 (m.p. 148–150°) melted at 146–148°. A mixture of the acetate with pregnanolone acetate (m.p. 97–99°) melted at 95–98°.

Non-ketonic Fraction

When the total non-ketonic fraction (8.6 g.) was dissolved in 750 ml. benzene, an amorphous fraction weighing 600 mg., with a melting range

[3] The melting points were determined with a Fischer-Johns apparatus and are uncorrected.

of 215–235°, settled out. The precipitate was crystallized from methanol yielding fine needles which melted at 234–236°. A mixture of the compound with pregnanediol-3(α),20(α) (m.p. 236–238°) melted at 234–237°. The soluble non-ketonic material remaining after removal of the preganediol-3(α),20(α) was chromatographed on a column containing 215 g. of a magnesium silicate-Celite mixture. Elution with benzene yielded crystals which on recrystallization from aqueous methanol yielded 80 mg. white needles with a melting point of 146–148°. A mixture of the compound with cholesterol (m.p. 144–145°) melted at 144–146°. Acetylation yielded crystals which melted at 113–115°. A mixture of the acetylated compound with cholesterol acetate (m.p. 114–115°) had a melting point of 113–115°.

Further elution with benzene yielded 90 mg. crystals with a melting point of 240–242°. A mixture of the compound with allopregnanediol-3(α),20(α) (m.p. 239–241°) melted at 240–242°. Acetylation of the product yielded crystals with a melting point of 136–138°. A mixture of the compound with allopregnanediol-3(α),20(α) diacetate (m.p. 138–140°) melted at 135–138°.

Elution with benzene, and benzene containing 1% ethanol, yielded the third batch of crystals with a melting point of 234–236°. A mixture of the compound with pregnanediol-3(α),20(α) (m.p. 236–238°) melted at 234–236°. Acetylation yielded crystals with a melting point of 160–163°. A mixture of the compound with pregnanediol-3(α),20(α) diacetate (m.p. 163–165°) melted at 160–163°.

ALLOPREGNANEDIONE-3,20[4]

Allopregnanedione-3,20 was administered to a 38-year-old man with rheumatoid arthritis. The dosage was 185 mg. given orally three times per day. The urine representing 3.16 g. of administered steroid was collected. The daily urine samples were extracted with butanol by the method of Venning (1937). The butanol residues were pooled and dissolved in 0.4 l. of water and 60 ml. of concentrated hydrochloric acid. Two hundred ml. of benzene was added and the mixture refluxed for 3 hours. The benzene was separated, fresh benzene added, and again the combined benzene extracts were evaporated to dryness and the total neutral fraction prepared.

The 17-ketosteroids were determined by the procedure of Holtorff and Koch (1940). The mean value of the 17-ketosteroids for a 12 day pretreatment period was 10.4 mg. per 24 hours. The mean value of the 17-

[4] Allopregnanedione-3,20 and pregnanedione-3,20 were generously supplied to us by Parke, Davis & Co. and Chemical Specialties Co., Inc. Allopregnanediol-3(β),20(α) and Δ16-pregnenol-3(β), one-20 were generously supplied to us by the Parke, Davis and Co.

ketosteroids during 6 days of treatment rose to 17.1 mg. per 24 hours.

Treatment of the total neutral extract with Girard's reagent *T* yielded 140 mg. in the ketonic fraction and 360 mg. in the non-ketonic fraction.

The non-ketonic fraction (360 mg.) was chromatographed on a column containing 7.2 g. of a magnesium silicate-Celite (1:1) mixture. Elution with benzene containing 1% ethanol yielded three fractions. The first eluate yielded 20 mg. crystals with a melting point of 244–247°. A mixture of the compound with allopregnanediol-3(α),20(α) (m.p. 242–247°) melted at 244–247°. Acetylation of this product yielded crystals with a melting point of 138–141°. A mixture of the compound with allopregnanediol-3(α),20(α) diacetate (m.p. 138–140°) gave a melting point of 138–140°.

The second eluate of 80 mg. had a melting point of 233–236° followed by a third fraction of 40 mg. with a melting point of 217–220°. A mixture of crystals from the third fraction with allopregnanediol-3(β),20(α) (m.p. 221–222°) gave a melting point of 221–222°. Acetylation of this compound yielded crystals with a melting point of 168–170°. A mixture of the acetate with allopregnanediol-3(β),20(α) diacetate (m.p. 167–168°) gave a melting point of 167–169°.

The second fraction containing crystals melting at 233–236° was treated with a 1% digitonin solution in 90% ethanol while hot and allowed to cool to room temperature. A precipitate separated readily and upon crystallization yielded 30 mg. of allopregnanediol-3(β),20(α) (m.p. 215–217°). The digitonin soluble fraction was hydrolyzed and upon crystallization yielded 20 mg. allopregnanediol-3(α),20(α) (m.p. 240–243°).

The ketonic fraction (140 mg.) was chromatographed on a column containing 4.2 g. of a magnesium silicate-Celite (1:1) mixture. No identifiable products were obtained.

PREGNANEDIONE-3,20

Pregnanedione-3,20 was administered orally to a 19-year old girl with rheumatoid arthritis. The dosage was 170 mg. given orally three times per day. Urine equivalent to 2.3 g. of administered steroid was collected.

The dried residues obtained from the butanol extracts were hydrolyzed and extracted by refluxing for two hours in a solution containing 200 ml. of water, 40 ml. of ethanol, 40 ml. of concentrated hydrochloric acid, and 300 ml. of benzene. The benzene extractions were repeated three times, the benzene extracts pooled, and evaporated to dryness. The total neutral fraction was prepared.

The 17-ketosteroids were determined by the procedure of Holtorff and Koch. The mean value for 17-ketosteroids for a 15 day pre-treatment period was 9.2 mg./24 hours. The mean value for 17-ketosteroids during five days of treatment rose to 14.5 mg. per 24 hours.

The total neutral extract (700 mg.) upon treatment with Girard's reagent *T* yielded 600 mg. in the non-ketonic fraction and 100 mg. in the ketonic fraction.

The non-ketonic fraction (600 mg.) was chromatographed on a column containing 18 g. of a magnesium silicate-Celite (1:1) mixture. Elution with benzene containing 1% ethanol yielded 180 mg. of crystals with a melting point of 239–241°. A mixture of the compound with pregnanediol-3(α)-20(α) (m.p. 236–238°) gave a melting point of 236–238°. The compound was acetylated by heating for one hour on a steam bath in a solution of pyridine and acetic anhydride. Crystals were obtained which melted at 163–164°. A mixture of the acetate with pregnanediol-3(α),20(α) diacetate (m.p. 163–165°) gave a melting point of 163–165°.

The ketonic fraction (100 mg.) was chromatographed on a column containing 3 g. of magnesium silicate-Celite (1:1) mixture. No additional products were identified.

Δ^5-PREGNENOL-3(β)-one-20[5]

Δ^5-Pregnenol-3(β)-one-20 was administered to five rheumatoid arthritic men and one man with non-specific myositis. The steroid was administered intramuscularly in oil or aqueous suspensions. The dosage varied from 200 mg. to 500 mg. per day. The urine collections represented 72.6 days equivalent or 25.5 g. of the steroid.

Determination of 17-ketosteroids was made by the procedure of Holtorff and Koch (1940). No significant change in 17-ketosteroid excretion was found.

The daily urine samples were extracted with butanol by the procedure of Venning (1937). The dried residues obtained from the butanol extracts were hydrolyzed and extracted by refluxing for 3 hours in a mixture of 2 l. of water, 0.2 l. of ethanol, 0.4 l. of concentrated hydrochloric acid, and 2 l. of benzene. The benzene extractions were repeated four times. The combined benzene extracts were evaporated to dryness and the total neutral fraction was prepared. Separation of the total neutral extract (6.0 g.) with Girard reagent *T* yielded 5.2 g. in the non-ketonic fraction and 0.9 g. in the ketonic fraction.

Treatment of the non-ketonic fraction (5.2 g.) with 1% digitonin in 90% ethanol gave a soluble fraction of 4.8 g. and a digitonin insoluble fraction of 0.35 g.

The non-ketonic digitonin precipitable fraction (0.35 g.) was chromatographed on a column containing 10.5 g. of a magnesium silicate-Celite

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[5] The Δ5-pregnenol-3(β)-one-20 was generously supplied to us by Chemical Specialties Co., Inc., Ciba Pharmaceutical Products, Inc. and Parke, Davis & Co.

mixture (1:1). Elution with benzene yielded crystals with a melting range of 115–130°. Recrystallization from aqueous methanol did not improve the melting point. The crystals were rechromatographed and on elution with benzene yielded crystals with a melting point of 145–147°. A mixture of the compound with cholesterol (m.p. 144–145°) melted at 143–145°. From the mother liquors a second crop of crystals was obtained which had a melting point of 60–64°.

Further elution of the non-ketonic digitonin precipitable fraction with benzene containing 1% ethanol yielded crystals with a melting point of 175–178°. A mixture of the compound with pregnanediol-3(β),20(α) (m.p. 184–187°) depressed the melting point to 150–160°. The 3(β)-diol was prepared by reduction of Δ16-pregnenol-3(β)-one-20[4] with sodium in boiling absolute ethanol. A mixture of the unknown compound with Δ5-pregnenediol-3(β),20(α) (m.p. 178–180°) melted at 176–179°. Acetylation of the compound yielded crystals with a melting point of 142–145°. A mixture of the compound with Δ5-pregnenediol-3(β),20(α) diacetate (m.p. 144–148°) melted at 142–146°.

The non-ketonic digitonin soluble material (4.8 g.) was chromatographed on a column containing 144 g. of a magnesium silicate-Celite mixture (1:1). Elution with benzene containing 1% ethanol yielded 300 mg. crystals with a melting point of 234–236°. A mixture of the compound with pregnanediol-3(α),20(α) (m.p. 234–236°) melted at 234–236°. Acetylation of the compound yielded crystals which melted at 163–167°. A mixture of the acetate with pregnanediol-3(α),20(α) diacetate (m.p. 163–166°) melted at 163–166°.

No identifiable products were obtained from the ketonic fraction.

DISCUSSION

Figure 1 illustrates some aspects of progesterone (I) metabolism. Progesterone by reduction of the double bond between carbon atoms 3 and 4 can give rise to pregnanedione-3,20 (II) and allopregnanedione-3,20 (III). Both of these saturated diones have been isolated from pregnancy urines, but not as direct metabolites of progesterone administration. After administration of these diones (II and III) we were unable to isolate unchanged material. These facts argue for the possibility that these compounds are present normally in exceedingly small amounts and are efficiently reduced further to the saturated hydroxy ketones IV, V, VI and VII. Saturated hydroxy ketones are in turn reduced to saturated diols VIII, IX, X and XI. Two of these saturated diols, that is pregnanediol-3(α),20(α) (VIII) and allopregnanediol-3(α),20(α) (X) are now shown to be metabolites of progesterone. The fact that allopregnanediol-3(β)-20(α) (XI) arises from allopregnanedione-3,20 (III) strongly indicates that

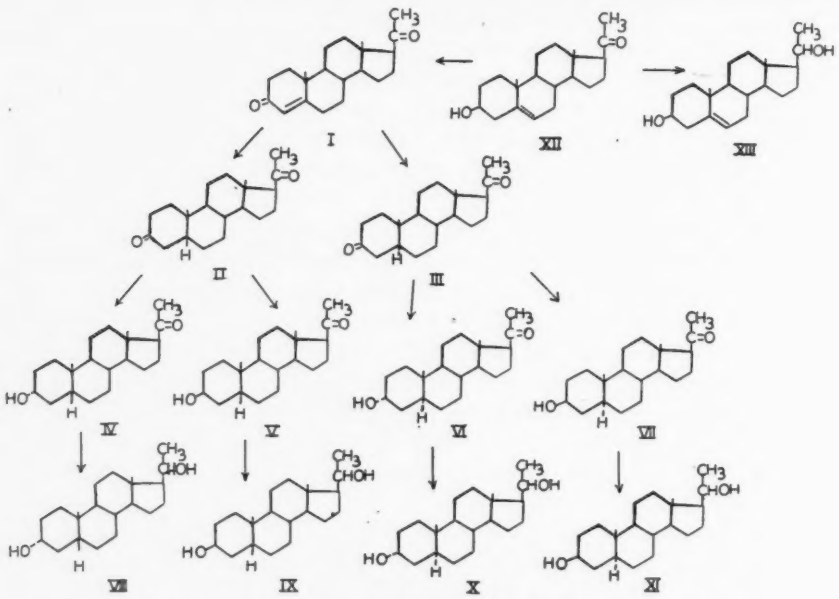

FIG. 1. Metabolism of progesterone and related compounds.

this 3(β) diol should also rise from progesterone. Thus far this relationship has not been demonstrated by direct *in vivo* experiments.

The finding of unchanged progesterone in the urine is probably due to the exceedingly large quantity of the steroid administered. It also must be remembered that these large quantities of progesterone were administered to rheumatoid arthritic patients. It is possible that the presence of unchanged progesterone in these urines may be related in some way to altered progesterone metabolism in such patients. Marrian (1950) has shown, for example, that rheumatoid arthritic patients excrete approximately twice the amount of pregnanediol-3(α),20(α) after the administration of progesterone as compared to the findings in normal individuals.

Δ5-Pregnenolone is converted to Δ5-pregnenediol-3(β),20(α) (XIII) and also to pregnanediol-3(α),20(α) (VIII), Figure 1, possibly by way of progesterone.

Table 1 summarizes the metabolites isolated from the urine.

It would appear that Δ5-pregnenolone bears a relationship to progesterone metabolism just as dehydroisoandrosterone does to Δ4-androstenedione metabolism. Reduction of both compounds to the corresponding Δ5-diol can occur as well as reduction to the saturated 3(α) hydroxyl derivative. Administration of dehydroisoandrosterone yields androsterone,

TABLE 1. IN VIVO METABOLISM OF PROGESTERONE AND RELATED COMPOUNDS

Steroid administered (amount in g.)	Steroids isolated	Amount mg.	Isolated %
Progesterone (24.26)	Progesterone	120	0.5
	Pregnanol-3(α)-one-20	150	0.6
	Pregnanediol-3(α), 20(α)	1460	6.0
	Allopregnanediol-3(α), 20(α)	90	0.4
Allopregnanedione-3,20 (3.16)	Allopregnanediol-3(β), 20(α)	70	2.2
	Allopregnanediol-3(α), 20(α)	40	1.2
Pregnanedione-3,20 (2.3)	Pregnanediol-3(α),20(α)	180	7.8
Δ^5-Pregnenol-3(β)-one-20 (25.5)	Δ^5-Pregnanediol-3(β),20(α)	120	0.5
	Pregnanediol-3(α),20(α)	300	1.2

etiocholanol-3(α),one-17 and Δ5-androstenediol 3(β),17(β) (Mason and Kepler, 1946; Miller, Dorfman and Miller, 1950).

Δ5-Pregnenediol-3(β),20(α) has been isolated from the urine of a woman with adrenal cortical cancer and from the urine of a girl with virilism by Schiller *et al.*, 1945, and from the urine of a boy with adrenal cancer (Hirschmann and Hirschmann, 1945). Δ5-Androstenediol-3(β),17(β) has been isolated from the urine of a boy with adrenal cancer (Hirschmann and Hirschmann, 1945) and in the urine of a woman with adrenal cortical cancer (Schiller *et al.*, 1945).

SUMMARY

Several aspects of the intermediate metabolism of progesterone *in vivo* have been studied by isolation of urinary metabolites.

Administration of progesterone led to the isolation in the urine of pregnanediol 3(α),20(α), allopregnanediol 3(α),20(α) and pregnanolone.

Administration of pregnanedione-3,20 yielded pregnanediol-3(α),20(α) in the urine.

Administration of allopregnanedione-3,20 yielded allopregnanediol-3(α),20(α) and allopregnanediol-3(β),20(α) in the urine.

Administration of Δ5-pregnenediol-3(β),20(α) led to the isolation in the urine of pregnanediol-3(α),20(α) and Δ5-pregnenediol-3(β),20(α).

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EFFECT OF PHYSIOLOGICAL AMOUNTS OF ESTROGEN ON THE SECRETION OF FOLLICLE STIMULATING AND LUTEINIZING HORMONES*

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THE problem of pituitary ovarian interrelationships has been investigated extensively during the last three decades and there seems to be general agreement that large quantities of gonadal hormones will suppress gonadotrophic hormone secretion. However, there is considerable controversy concerning the quantitative aspect of the effect of gonadal hormones on the secretion of gonadotrophins. A most important consideration in this problem is whether or not estrogen inhibits the production of gonadotrophins when within physiological limits, and whether estrogen stimulates the production and/or release of gonadotrophic hormone. Some confusion results from the failure to distinguish between the gonadotrophic content of the pituitary and the amount of hormone being secreted by the gland. Although much work has been done on the effect of estrogen upon the amount of hormone within the gland, this may not always be a valid index of the secretion rate of the hormone.

The decrease in gonadotrophic hormone secretion by estrogen administration was demonstrated by Moore and Price (1930), Meyer, Leonard, Hisaw and Martin (1930), Hisaw, Fevold, Foster and Hellbaum (1934) and others. Although Meyer, Leonard, Hisaw and Martin (1932) were able to show a decrease in the gonadotrophic hormone content of the pituitary of estrogen-treated rats, Lauson, Heller and Sevringhaus (1937, 1938) reported that the pituitaries of rats treated with large amounts of estrogen did not exhibit a decrease in gonadotrophin. Heller and Heller (1939) and Heller, Heller and Sevringhaus (1942) maintain, " . . . estrogen in amounts that already overstep the physiological bounds necessary for some organs, do not decrease pituitary potency in the rat, or gonadotrophic excretion in the menopausal female." The view that estrogen is not the physiological mechanism for the inhibition of gonadotrophic hormone secretion is also supported by Jungck, Heller and Nelson (1947), Lipschutz,

Received for publication February 28, 1951.

* This investigation was supported in part by a research grant from the National Institutes of Health, U. S. Public Health Service, and from the Wisconsin Alumni Research Foundation.

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Iglesias, Brussone, Hemerez and Penaranda (1948), Greep and Jones (1950 b), and others.

There also is not complete accord concerning the effect estrogen has on the secretion of luteinizing hormone. The release of LH by estrogen as evidenced by the luteinization of ovarian follicles was demonstrated by Hohlweg (1934), Hisaw, Fevold, Foster and Hellbaum (1934) and confirmed by others. Merckel and Nelson (1940) maintained the corpora lutea of rats for three weeks by daily injections of estrone. More recently, Hellbaum, Funnell and Keaty (1949) administered large doses of estrogenic preparations to menopausal women and were able to demonstrate LH in the urine where previously only FSH was found. The pituitaries of estrogen-treated rats have been shown to contain more LH than pituitaries of control animals (Lane and Hisaw, 1934). This was not confirmed, however, by Laquer and Fluhmann (1942) and Hellbaum and Greep (1943, 1946) who treated female rats with steroids and found a decrease in the amount of LH in the pituitaries. Greep and Jones (1950 b) grant that an elevation of the estrogen level in intact animals may under some circumstances effect a release of whatever LH may be present in the gland but believe, " . . . the fundamental effect of estrogen on the pituitary is to reduce the synthesis of LH."

These varied results indicate that under some conditions gonadal hormones increase, and under other conditions decrease the gonadotrophic activity of the pituitary, but the exact relationships remain obscure. The purpose of the experiments in this paper was to determine whether estrogen in physiological doses inhibits FSH secretion in normal female rats, and whether, under some conditions estrogen alters the secretion of LH by the pituitary.

MATERIALS AND METHODS

The following experiments were designed to demonstrate the effect of varying doses of estrogen on the pituitary gonadotrophic hormone activity in immature and in adolescent female rats. For the immature rats (Group A), thirty-day old females of the Sprague-Dawley strain weighing 70 grams were used. These rats have closed vaginas, ovarian weights averaging 26 mg. and uterine weights averaging 46 mg. The unstimulated condition of the uterus at 30 days is indicated by its failure to decrease in weight following ovariectomy at this age.

For the older group of rats (Group B), fifty-five day old females of the Sprague-Dawley strain weighing 160 grams were used. These animals have open vaginas, an average ovarian weight of 52 mg. and an average uterine weight of 287 mg.

Alpha-estradiol was administered to the rats of Group A and Group B at eight different doses. The estrogen was dissolved in corn oil and 0.1 cc. of the oil was injected subcutaneously daily for ten days. As a control measure, rats of Group A and B were injected with corn oil alone. On the 11th day, the rats were killed with illuminating gas, and ovarian and uterine weights were determined. The ovaries were examined for corpora

lutea and follicles, and the uterus for the presence of intra-uterine fluid. At the time that the rats in Group B were autopsied, the pituitaries were removed and assayed for gonadotrophic hormone content. The fresh pituitaries from the rats in each of the nine groups were homogenized and suspended in normal saline. The pituitary homogenate was injected subcutaneously twice daily for four and one half days into 21-day old female rats of the Holtzman strain. Three assay rats were used in each group, so each rat received the pituitary equivalent of three adult rats. On the 5th day, the assay rats were killed, and the ovarian and uterine weights were determined. The ovaries were examined for follicles and corpora lutea, and the uterus for intra-uterine fluid.

Females of Groups A and B were ovariectomized and injected with alpha-estradiol at different doses according to the procedures described above for the intact rats of the same age. To serve as controls, rats in both groups were also injected with corn oil alone. Vaginal smears were taken on the 11th day. The rats were then killed, and uterine and thymic weights determined.

RESULTS AND DISCUSSION

The results of estrogen administration to immature female rats (Group A) are shown in Table 1 and in Figure 1. It it seen that there was a decline in ovarian weight from the control value when alpha-estradiol was injected in the smaller amounts (0.002 μg.–0.009 μg.). This was followed by an increase in ovarian weight at the higher estrogen levels (0.012 μg.–0.02 μg.),

TABLE 1. INTACT AND OVARIECTOMIZED IMMATURE FEMALE RATS INJECTED WITH ALPHA ESTRADIOL DAILY FOR TEN DAYS

Intact Females				Ovariectomized Females		
No. rats	Dose (μg./day)	Ovaries (mg.)	Uterus (mg.)	No. rats	Dose (μg./day)	Uterus (mg.)
8	none	41 (25–50)	185 (101–254)	6	none	52 (43–65)
4	.002	36 (20–42)	176 (96–242)	6	.01	55 (45–70)
3	.005	26 (22–36)	156 (97–188)	8	.02	65 (50–82)
4	.0075	23 (21–30)	104 (83–146)	5	.03	73 (63–80)
4	.009	19 (12–28)	60 (55–76)	4	.04	101 (83–114)
5	.012	21 (18–26)	70 (64–95)	4	.05	130 (94–151)
9	.020	30 (22–40)	108 (94–138)			
4	.030	16 (10–27)	120 (102–162)			
4	.05	16 (12–30)	168 (123–285)			

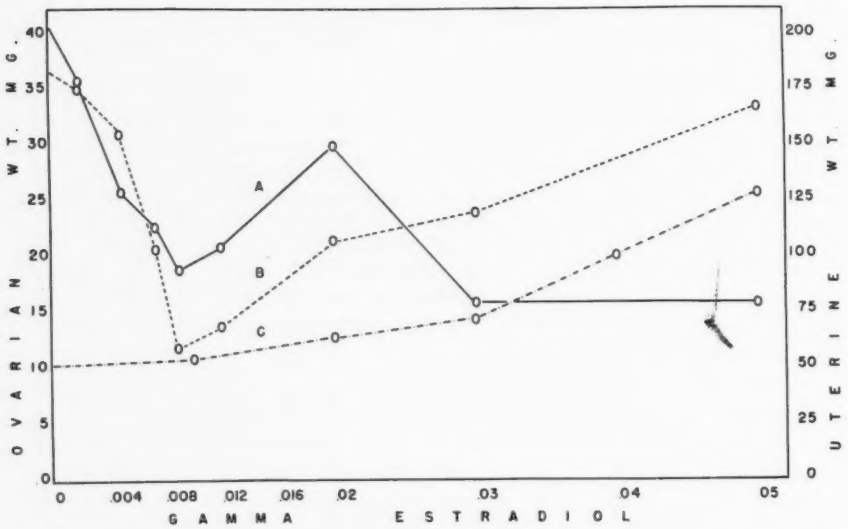

FIG. 1. The effect of estrogen on the ovarian and uterine response in intact and ovariectomized immature rats. (A) ovaries, (B) uteri of intact rats, (C) uteri of ovariectomized rats.

and a second decrease in weight when the estrogen was administered in larger amounts (0.03 μg.–0.05 μg.). At both the first and second decline in ovarian weight, the ovaries were not only below the normal 40-day ovarian weight, but less than the 30-day ovarian weight, which demonstrates an atrophy from the 30-day condition.

The uterus serves as an index of the blood level of estrogen, both from exogenous and endogenous sources. At the first ovarian decline there was a decreased secretion of estrogen correlated with the atrophy of the ovaries. The increased weight of the uterus in the animals with hypertorphied ovaries is probably due mainly to greater secretion of estrogen by the ovaries.

The castrated immature rats injected with estrogen showed slight uterine stimulation when alpha-estradiol was administered in doses which caused the first ovarian decline (Figure 1). The uterine hypertrophy at the second ovarian decline was also small and well below that found in the intact rats of this age. Cornified cells were not found in the vaginal smears of the rats receiving any of the doses of estrogen.

Based on the data from 8 rats in each group, it was observed that the thymus increased in weight from 268 mg. to 444 mg. between 30 and 40 days. Since the weight of the thymus of 40-day old females ovariectomized for ten days was 496 mg., it appears the estrogen normally present at this

age has little effect on the thymic weight. When 0.04 μg. of alpha-estradiol was administered daily for ten days to ovariectomized females, the weight of the thymus was 480 mg. This indicates no involution of the thymus induced by the estrogen at the level causing the second decline in immature rats.

It is concluded that at this age the gonadotrophic hormone secretion is decreased by estrogen in amounts well within physiological limits. This is in agreement with findings of Byrnes and Meyer (1951) that the post-castration hypersecretion of FSH was inhibited in immature rats in parabiosis with normal littermates by estrogen in doses causing no increase in uterine weight.

The time factor appears to be important in the inhibition of gonadotrophins with estrogen in ovariectomized animals. After the pituitary has undergone post-castration hypersecretion, more estrogen is needed to inhibit the production of gonadotropes than when estrogen replacement is begun immediately after ovariectomy (Byrnes, 1951). This may explain, in part,

TABLE 2. INTACT AND OVARIECTOMIZED ADOLESCENT FEMALE RATS INJECTED WITH ESTRADIOL DAILY FOR TEN DAYS; 21-DAY OLD FEMALES INJECTED WITH PITUITARIES FROM THE HORMONE TREATED INTACT RATS

Intact females				Rats given pituitaries of estrogen treated intact females		Ovariectomized females		
No.	Dose (μg./day)	Ov. wt. (mg.)	Uterine wt. (mg.)	No.	Ov. wt. (mg.)	No.	Dose (μg./day)	Uterine wt. (mg.)
9	none	66.7 (48–88)	356 (290–500)	3	21.3 (20–24)	4	none	89 (83–95)
9	.015	63.5 (56–76)	333 (290–418)	3	15.3 (14–16)	7	.02	93 (70–125)
9	.025	50.3 (42–67)	292 (205–365)	3	17.3 (14–20)	6	.025	112 (107–120)
9	.035	59.3 (46–72)	348 (255–550)	3	35.3 (25–50)	3	.04	139 (100–167)
9	.050	65.7 (61–92)	326 (258–420)	3	24.6 (20–27)	6	.075	184 (150–235)
9	.075	69.0 (60–76)	380 (265–490)	3	46.6 (40–53)	6	1.25	199 (181–235)
9	.10	59.3 (45–85)	305 (240–345)	3	21.3 (15–31)	10	1.05	220 (188–280)
6	.125	58.1 (45–80)	325 (242–515)	3	14 (11–15)			
9	.175	55.6 (47–64)	383 (310–480)	3	17 (17–25)			

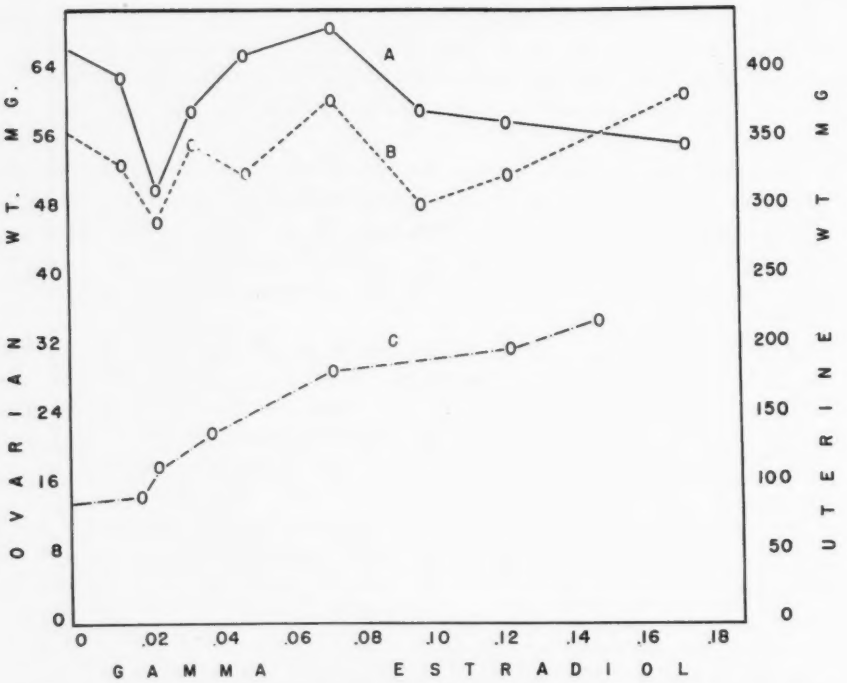

FIG. 2. The effect of estrogen on the ovarian and uterine response in the intact and ovariectomized adolescent rats. (A) ovaries, (B) uteri of intact rats, (C) uteri of ovariectomized rats.

the large and unphysiological amounts of estrogen reported necessary to inhibit gonadotrophin secretion in castrated and menopausal women (Heller, Heller and Sevringhaus, 1942).

In Table 2 and in Figure 2, the ovarian response to estrogen in the adolescent females (Group B) is seen to follow the same pattern observed in the immature rats (Group A). At the first and second decreases in ovarian weight, the ovaries weighed less than those of normal 65-day old rats. The ovarian weight obtained in the adolescent rats with alpha-estradiol at 0.075 μg. was *greater* than that of intact females of the same age. In contrast, the ovarian weight of immature rats given doses of estrogen between those which produced the first and second decreases in ovarian weight was *less* than the ovarian weight of intact rats of this age. The larger ovaries observed in both the immature and adolescent females treated with intermediate doses of estrogen was due primarily to the increased number and size of corpora lutea.

The uterine weights in Group B (Figure 2) reflected the amount of exog-

enous estrogen together with any estrogen secreted by the ovaries. Accompanying the ovarian hypertrophy in the rats administered 0.075 μg. of alpha-estradiol, there was greater growth of the uterus. This was due mainly to an increased secretion of estrogen by the ovaries since it was followed by a decrease in uterine weight in spite of a higher dose of estrogen being administered. There was an apparent drop in the estrogen level in the rats injected with 0.05 μg. of estradiol, although this was not accompanied by a decline in ovarian weight.

It is to be emphasized that in both the immature and the adolescent females administration of estrogen at very low doses produced a marked *decrease* in uterine weight. In none of the groups of the injected immature females was the uterine weight as great as in the uninjected controls. In the older females, the uterine weight was above the controls only in the group with the maximal ovarian hypertrophy and in the group receiving the greatest amount of exogenous estrogen.

The vaginal smears of the rats in Group B were essentially unchanged from normal when the estradiol was administered at doses less than 0.05 μg. per day. At doses of the estrogen as high as 0.175 μg. per day, there was still some "cycling."

The results obtained by administering alpha-estradiol to ovariectomized fifty-five day old females for ten days are found in Table 2 and in Figure 2. The uterine stimulation was slight when estrogen was injected at the level of the first ovarian decline. There was a considerable increase in the weight of the uterus when alpha-estradiol was administered at the dose which caused the second ovarian decline. The uterine weight was, however, less than that of intact females of this age. There were cornified cells in the vaginal smears of the rats injected with 0.075 μg. and larger amounts of alpha-estradiol. It is concluded that in the adolescent female rats, the inhibition of gonadotrophic hormone secretion at both the first and second decreases in ovarian weight was brought about by estrogen given in physiological amounts.

The data from the assay of the gonadotrophic hormone in the pituitaries of the injected adolescent females (Group B) is presented in Table 2 and Figure 3. The ovarian weight of the uninjected assay rats averaged 17 mg. By comparing the curves of the ovarian responses in Figures 2 and 3, it is seen that there is a general similarity. The ovaries of the assay rats injected with the pituitaries of adolescent females showing the first and second decreases in ovarian weight (i.e. 0.025 μg. and 0.125 μg. estradiol) were small and did not contain corpora lutea. The uteri of the rats at these stages contained a large amount of intra-uterine fluid, as is characteristic in the presence of small amounts of estrogen. The ovaries of the assay rats were largest in those injected with the pituitaries of the females with

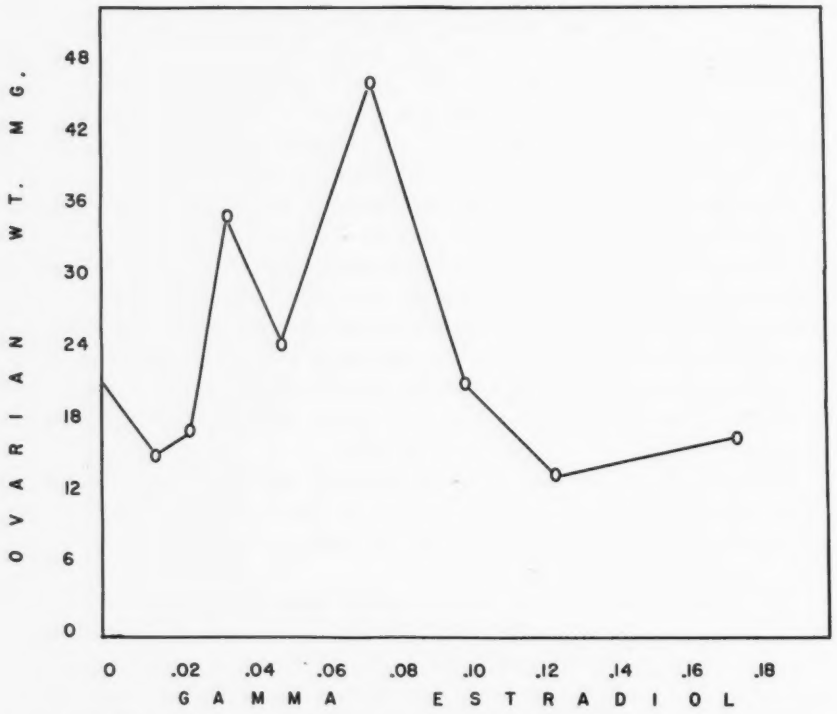

FIG. 3. The ovarian response of 21-day old rats injected with the pituitaries of adolescent rats administered estrogen.

the greatest ovarian hypertrophy. The ovaries of these rats contained numerous large corpora lutea.

It was previously pointed out that there was a drop in the uterine weight in the rats injected with 0.05 μg. of alpha-estradiol. The reason for this remains obscure. It is of interest that although this drop in interine weight was not accompanied by smaller ovaries, it did correlate with a decreased amount of gonadotrophins in the pituitary. This indicates that the uterine weight of the rat correlates more closely with the pituitary gonadotrophic content than does the ovarian weight. A possible explanation of this is that the ovaries do not respond to an altered hormonal balance by changes in weight as rapidly as the uterus.

In comparing the ovarian weights of the donors and the recipients, the latter seem to constitute a more sensitive index of the amount of pituitary gonadotrophic activity. It is to be emphasized, however, that the ovaries of the donors reflect the gonadotrophic activity for the entire period of injection, while the ovaries of the recipients measure the gonadotrophic hormone present in the pituitary of the donor at the time of the autopsy. The close

correlation between the uterine and ovarian weights of the donors and the ovarian response of the recipients suggests that, in these experiments, the level of gonadotrophin in the pituitary rises and falls as the secretory rate increases and decreases.

On the basis of the data presented in this paper, it is postulated that the first ovarian decline was caused by the secretion of smaller amounts of follicle stimulating hormone. In both the immature (Group A) and the adolescent females (Group B), the lowered FSH secretion was associated with small ovarian weights. When the estrogen was injected in larger amounts, there was an increased secretion of luteinizing hormone by the pituitary. The LH caused a greater production of estrogen by the ovaries, which in turn produced further uterine hypertrophy. The LH also caused luteinization of the follicles and an increase in ovarian weight. This effect was particularly marked in the assay rats injected with the pituitaries of females administered an intermediate amount of estrogen (i. e. 0.075 ug. estradiol). In both Group A and Group B, less LH was secreted when the estrogen was administered in the largest doses. This resulted in the second decrease in ovarian weights.

It is suggested that the decrease in FSH activity of the pituitary obtained with estrogen at a low level, followed by the increased secretion of LH at a moderate level might account for conflicting reports in the literature on the effect of exogenous estrogen upon gonadotrophic activity. It is clear that in any study of the effect of estrogen on gonadotrophic hormones, data from estrogen administration at only one dose would be an inadequate basis for a comprehensive interpretation.

The time relationships involved in experiments such as these may be a very important consideration. It is possible, for example, immediately following the release of luteinizing hormone by the pituitary under the influence of estrogen the pituitary *content* of this hormone would be low. Bradbury (1947) administered estrogen to immature female rats and reported an increase in ovarian weight in 72–120 hours, but a decrease in gonadotrophic hormone content of the pituitary in 72–96 hours. Continued administration of estrogen might stimulate the *production* of the hormone as well as its *secretion*, so that an assay of the pituitary after ten days shows luteinizing hormone to be present in large amounts.

On the other hand, it appears prolonged estrogen treatment again decreases the LH activity of the pituitary (Fevold, Hisaw and Greep, 1936). Greep and Jones (1950 a) gave estrogen to female rats for 45 days and obtained a decrease in ovarian weight in the animals receiving estrogen in low doses. In contrast to the experiments reported in this paper, there was no evidence of estrogen in moderate amounts causing an increased secretion or production of LH by the pituitary. Since their experimental procedures were similar to those reported here, it seems probable the pituitary

was exhausted of LH under long term estrogen administration.

The cyclic nature of the reproductive activity in the rat can be accounted for on the basis of slight changes in the estrogen level in the blood which reciprocally affects the secretion of FSH and LH. It is postulated that at no time in the cycle does either of these hormones exist in the complete absence of the other. When the level of estrogen is lowered through decreased secretion by the luteinized follicle, and through inactivation by the liver, the pituitary secretes larger amounts of FSH. It is probable that some LH is also produced, since Cutuly, McCullagh and Cutuly (1937), Greep (1940) and Biddulph and Meyer (1946) demonstrated LH as well as FSH is secreted by the pituitary of an ovariectomized rat. Under the influence of FSH and LH, the ovaries produce follicles which secrete estrogen. When the level of estrogen is still low, FSH secretion is decreased. As the estrogen in the blood reaches moderate levels, the secondary sex organs are stimulated, and the pituitary secretes increased amounts of LH. Under the influence of LH, the ovaries produce larger quantities of estrogen which cause further stimulation of the accessories. When the estrogen level of the blood becomes high, the LH output is reduced. Since FSH as well as LH is now low, the estrogen secretion by the ovaries declines rapidly and the cycle is completed. This hypothesis is essentially in agreement with that proposed by Hisaw, Fevold, Foster and Hellbaum (1934) and Fevold, Hisaw and Greep (1936).

Other interpretations of pituitary-ovarian interrelationships are presented by Jungck, Heller and Nelson (1947), Jungck (1948), and Greep and Jones (1950 b).

The scheme presented in this paper omits the role of progesterone. Byrnes, Meyer and Finerty (1950) demonstrated that the gonadotrophic (FSH) inhibiting activity of progesterone compared with alpha-estradiol in the ratio of about 155,555:1 in parabiotic female rats. These authors also report progesterone does not potentiate nor antagonize alpha-estradiol in the inhibition of gonadotrophic hormone (FSH) secretion. It seems unlikely progesterone *per se* plays an important part in inhibiting gonadotrophic secretion in the estrous cycle of the rat.

In comparing the amount of estrogen needed to effect the pituitary secretion of gonadotrophic hormone in the immature and the adolescent females, it was found that greater amounts of hormone were required in the older animals. The quantity of estrogen required to inhibit FSH, as based on the amount causing the first ovarian decline, increased from the immature to the adolescent rats by a factor of 2.77. The estrogen needed to stimulate the secretion of LH, based on the peak of ovarian stimulation following the first ovarian decline increased by a factor of 3.75. It is to be remembered, however, that the older animals are heavier and the increase in the quantity of estrogen required could be due to a dilution.

Using the body weights of the animals at the time of autopsy, we find the weights of the two groups differed by a factor of only 1.43. The dilution of the estrogen does not appear to have an important influence on the degree of uterine response to a given amount of estrogen. In both the immature and the adolescent rats, an increase in uterine weight of 50 or 75 mg. was produced by the same doses of estradiol. It seems the amount of estrogen required to affect the secretion of gonadotrophic hormone increased in the older animals to an extent which cannot be accounted for on the basis of a dilution alone. The greater amount of estrogen required can be accounted for on the basis of a decreased sensitivity of the older pituitary to estrogen.

Ovaries of immature animals have been found to contain estrogen in levels comparable to that of the adult (Zephiroff and Dobrovolskaya-Zavadskaya, 1940). Estrogen is secreted by the immature ovary at a level which does not cause uterine growth, but which is sufficient to keep the gonadotrophic hormone secretion at a low level (Martins, Rocha, and Silva, 1931). Castration of an immature rat releases the pituitary from estrogen control and a hypersecretion of FSH ensues as is shown by parabiotic studies (Kallas, 1930 and others). As the animal matures, it appears the pituitary becomes less sensitive to the estrogen being elaborated by the ovaries. The secretion of FSH is, therefore, not inhibited until the estrogen level in the blood is higher than that which inhibits FSH secretion in the immature animal. This results in the secretion of FSH in larger amounts than in immature animals, and ovarian follicular development follows. The developing follicles secrete estrogen in quantities adequate to stimulate the secondary sex organs and to cause the release of LH from the pituitary, and the adult estrous cycle continues as described earlier.

SUMMARY

Alpha-estradiol was injected daily into immature and adolescent female rats over a ten day period. The weight and qualitative response of the ovaries and uteri indicate the secretion of FSH was decreased by estrogen in amounts which were very small. The administration of alpha-estradiol at somewhat higher doses caused the increased secretion of LH, as determined by the luteinization of the follicles and the greater production of estrogen. Alpha-estradiol at the highest doses decreased the pituitary secretion of LH and the estrogen production by the ovaries. All of the responses to estrogen were at levels of the hormone which were small and within "physiological" limits.

It was demonstrated by assaying the pituitaries of the adolescent rats injected with estradiol that there was a very close correlation between the gonadotrophic hormone content of the pituitary and the hormone which had been secreted into the blood stream.

The evidence presented substantiates the hypothesis that the estrous cycle in the rat is mediated through slight fluctuations in the estrogen level in the blood. It appears FSH secretion is greatest when the estrogen is at very low levels and more LH is produced when the estrogen is present in slightly larger amounts.

The data indicate proportionately more estrogen was required to decrease FSH secretion and to stimulate LH production in adolescent than in immature female rats. It is postulated that the increased refractiveness of the pituitary of the adolescent to estrogen results in higher levels of FSH and is an important factor in the attainment of sexual maturity.

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THE INFLUENCE OF AGE, STRAIN, CASTRATION AND ESTROGENS UPON HEPATIC STORES OF VITAMIN A IN THE MOUSE*

BENJAMIN RUSH** AND B. L. TRUSCOTT***
WITH THE TECHNICAL ASSISTANCE OF NORAH GRANT RUSH

ABUNDANT evidence supports the presence of vitamin A-estrogen associations in the reproductive system. Vitamin A-deficient rats show a persistent cornification of the vaginal epithelium which resembles the vaginal epithelium of rats during estrus (Evans and Bishop 1922). Daily administration of large doses of carotene to female rats results in the continuous presence of a diestrous vaginal smear (Sherwood, Brend and Roper, 1936). The total, hepatic vitamin A is higher in female than in male rats when on either a normal diet or a diet supplemented with excess vitamin A (Sampson and Hackford, 1945; Callison and Knowles, 1945; Brenner et al., 1942). Female rats have a greater tenacity than male rats. The amount of vitamin A retained in the livers of castrate, A-deficient, male rats is similar to that of intact females (Bult and Sorgdrager, 1934).

This investigation is directed towards determining the normal level of hepatic vitamin A in the mouse and determining the influence of castration, estrogen injection, age, sex and strain upon hepatic vitamin A.

METHODS

The animals used were first generation hybrid mice designated CC_1, CC_2, AC_1 and AC_2[#] or mice of inbred strains C_{57}, A, C_3H and CBA. All experimental animals were fed on Purina "Fox Chow" exclusively. The mice were housed in an air conditioned room (Temperature 70 to 74°F., Humidity 50 to 60%).

One hundred and twenty-five mice were divided into groups according to sex, age and strain. Male and female mice were selected from some of these groups and gonadectomized, or gonadectomized and given 2 mg. pellets of estrone subcutaneously. At

Received for publication March 15, 1951.

* This investigation was supported in part by a grant from the James Hudson Brown Fund of Yale University School of Medicine, and by grants from the National Cancer Institute, United States Public Health Service, and The Jane Coffin Childs Memorial Fund administered by W. U. Gardner.

** The data, in part, was presented in a portion of a thesis submitted to the Yale University School of Medicine. Present address, Yale University, Department of Surgery, Division of Oncology.

*** Present address, University of North Carolina, Department of Anatomy.

[#] $CC_1 = (C_{57}\ ♀ \times CBA\ ♂)$; $CC_2 = (CBA \times C_{57}\ ♂)$; $AC_1 = (A\ ♀ \times C_3H\ ♂)$; $AC_2 = (C_3H\ ♀ \times A\ ♂)$

varying intervals mice were sacrificed, their livers were removed and weighed and the hepatic tissue analyzed for vitamin A content, employing the antimony trichloride reaction (Carr and Price, 1936), using a Lumetron photoelectric colorimeter calibrated with the U.S.P. vitamin A reference standard. The procedure followed was that of Gallup and Hoefer (1946).

To evaluate the results of these experiments, the value of "t" was calculated in compared groups. The probability of "t" was derived from standard tables for small groups. Results were considered significantly different when the probability of the null hypothesis was less than .01.

EXPERIMENTAL DATA

Observations on 17 intact female mice revealed that the mean vitamin A per gram of the hepatic tissue was 337 units. The mean vitamin A per gram of liver from 25 intact male mice was 287 units. The values were lower in the male than in the female but the difference was not large enough to be statistically significant. The age range of the male and female mice in this group was 60 to 70 days.

TABLE 1. HEPATIC VITAMIN A IN INTACT MICE

Strain	No. of animals	Age (days)	Animal wt.	Liver wt.	Total A	Vit. A/gm.	*t*	*P.*
CC_1	8	66	24.3	1.690∓.161	461∓66.6	272∓44.6	1.9	> .05
AC_1	8	70	23.2	1.679∓.300	523∓105	315∓53.9	1.1	> .1
A	9	67	17.6	1.078∓.139	368∓49.4	356∓84.8	.63	> .5
C_{57}	7	76	21.9	1.264∓.314	413∓101	332∓62.9	1.4	> .1
C_3H	8	62	18.7	1.416∓.109	404∓98.5	286∓51.8	.33	> .5
CBA	8	61	20.7	1.357∓.139	395∓61.4	296∓62.6		

There was no significant difference in the vitamin A per gram contained in the hepatic tissues of 2-month old mice of the inbred strains A, CBA, C_{57} and C_3H, and the first generation hybrids designated CC_1 and AC_2 (Table 1). The hybrid mice are larger and have significantly larger livers

TABLE 2. HEPATIC VITAMIN A IN NORMAL MICE AT DIFFERENT AGES

No. of animals	Strain	Age (days)	Wt. (gms.)	Liver wt. (gms.)	Vit. A/gm.	*t*	*P*
6	CC_1	29	16.3	1.032	107±18.9	7.9	< .01
8	CC_1	66	24.3	1.690	272±44.6	5.1	< .01
4	CC_1	140	27.9	1.781	577±67.9	6.0	< .01
4	CC_1	358	34.9	—	1364±216		
4	A	47	15.1	.917	170±22.1	5.7	< .01
9	A	67	17.6	1.078	356±84.8	3.3	< .01
4	A	83	18.5	1.099	549±87.5		
4	C_{57}	52	21.6	1.485	167±20.0	5.5	< .01
7	C_{57}	76	21.9	1.264	332±62.9		

than mice of the inbred strains but the amount of vitamin A per gram of hepatic tissue is approximately the same.

The concentration of vitamin A in the livers of mice increases with advancing age (Table 2). This is true both in mice of the inbred strains and the hybrid groups. The increase in concentration approximated a linear progression. Graph I shows the rise in hepatic vitamin A with increase in age in intact CC_1 mice.

Castrated mice, whether male or female, show an increased storage of vitamin A in the liver (Table 3). The increase is significant within 6 weeks following castration and the accumulation of vitamin A in the livers

TABLE 3. HEPATIC VITAMIN A IN CASTRATE MICE

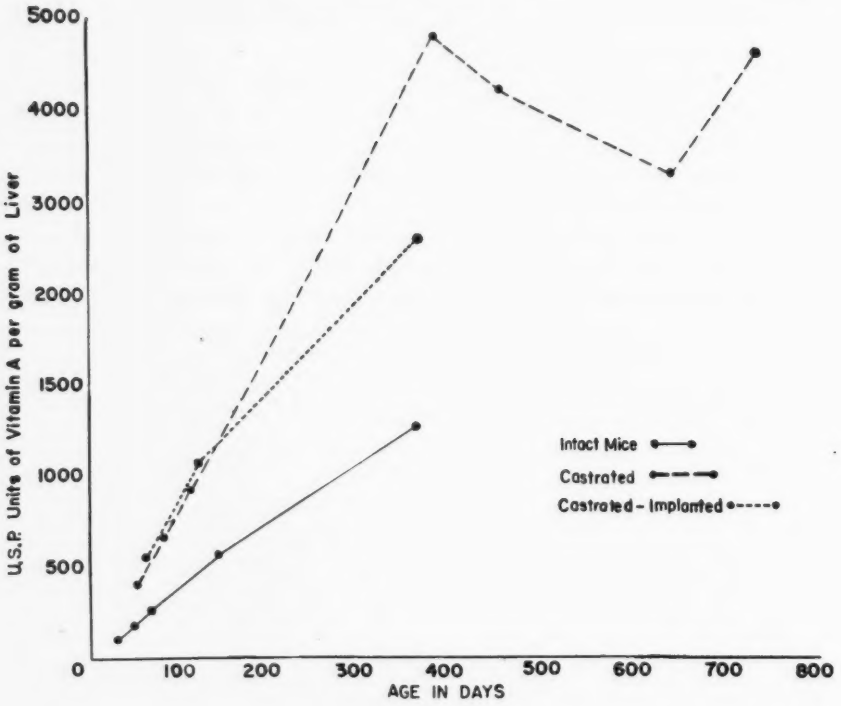
No. of animals	Period of castration (wks.)	Age (days)	Weight (gm.)	Liver wt. (gm.)	Vit. A/gm.	t	$P.$
6	2	62	26.8	—	403 ± 254	2.1	> .05
7	4	84	26.4	1.373	655 ± 156	2.0	> .05
7	6	109	27.0	—	867 ± 206	7.8	< .01
7	36	395	—	—	2258 ± 380	4.8	< .01
4	45	380	31.9	2.364	4813 ± 1060	.9	> .40
5	52	450	34.3	1.791	4210 ± 540	2.5	> .05
5	78	643	—	—	3285 ± 167	.3	> .50
7	94	725	33.8	1.723	4633 ± 1190		

* Standard deviation given after ±.

of castrated mice continues at a rate significantly more rapid than in intact mice (compare data in Tables 2 and 3). One year after castration the vitamin A per gram in the hepatic tissue of the castrated mice reaches a maximum which is maintained for at least another 12 months. Hepatic tissue of mice, castrated for 12 months, contained a concentration of vitamin A three times the level found in intact mice the same age.

The average vitamin A per gram in the hepatic tissue of 8 male mice a year or more after castration was 4,448 units. The hepatic tissue of 7 ovariectomized females a year or more after castration contained 5,222 units of vitamin A per gram. The difference was not statistically significant.

Graph I illustrates the rise in hepatic vitamin A in castrated mice plotted against age. The group of castrates 395 days old were omitted from the graph because they were castrated at the age of 143 days, much later than the other groups. The level of hepatic vitamin A in castrate mice reaches a plateau at 45 weeks. As the values for "t" show (Table 3) there is no significant increase or decrease at 52, 78, or 94 weeks.



GRAPH I

Male and female mice that were gonadectomized and had subcutaneously implanted 2 milligram pellets of estrone showed an increase in hepatic vitamin A (Table 4) which was the same as the increase in the castrated but otherwise untreated mice (Table 4 with Table 3).

Autopsy of these mice, done when the hepatic vitamin A was determined, showed fragments of the subcutaneous pellets of estrone still present: furthermore the pubic ligament and the accessory sexual organs showed the stigmata of estrogen effect.

Mature mice weighing more than 23 grams at the time of castration,

TABLE 4. HEPATIC VITAMIN A IN CASTRATED MICE WITH PELLETS OF ESTRONE IMPLANTED SUBCUTANEOUSLY

No. of animals	Period of castration (wks.)	Age (days)	Weight (gm.)	Vit. A/gm.	t	P
6	2	71	21.5	538∓254	2.2	> .05
3	6	120	24.0	1045∓273		
7	36	367	—	2586∓117	7.7	< .01

gained an average of 2.2 grams in two weeks and 3.6 grams in six weeks following castration. On the other hand, mature, castrated mice weighing more than 23 grams which received subcutaneous implantation of estrone pellets, gained only 0.43 gram in the two weeks following castration and at the end of six weeks had lost 1.7 grams compared to their weight at castration.

DISCUSSION

Hepatic vitamin A in intact mice on a normal diet increased with advancing age and demonstrates the necessity of "age" controls in experimenting with factors affecting vitamin A levels. A difference of only 20 days in the age of the animals resulted in a significant difference in the hepatic concentration of vitamin A (Table 2).

The accumulation of vitamin A in the liver with increase in age was accelerated in castrated mice. The administration of large amounts of estrone to the castrates by subcutaneous implantation of estrone pellets did not alter the accumulation of vitamin A significantly. After a year of treatment the castrates implanted with estrone showed a rise in hepatic vitamin A similar to that found in castrated mice. It is difficult to interpret this last result due to the unphysiologically large amounts of estrogen administered.

An increase in the vitamin A stores of the liver in mice on a normal diet can depend on one or more of several factors: an increase in absorption of the vitamin from the gut; a decrease in utilization of vitamin A by the tissues; an impairment of the ability of the liver to mobilize its stores of vitamin A and a decrease or failure of the organism either to destroy excess amounts of vitamin A or to excrete them. Which of these factors is responsible for the changes in hepatic vitamin A observed in these experiments cannot be stated.

SUMMARY

Inbred and hybrid strains of mice were selected. Some of these mice were intact, some were castrated and a few were castrated and at the same time received 2 mgm. pellets of estrone subcutaneously. At varying intervals the mice were sacrificed and observations made on their weight, liver weight, and content of vitamin A per gram of liver tissue.

The mean concentration per gram of hepatic tissue was 337 units in females and 287 units in males. The difference was not statistically significant. There was no difference in the concentration of vitamin A in the livers of inbred and hybrid mice of the same age. The vitamin A content of the livers of normal mice increased with age. The vitamin A content of the livers of castrated mice increased with age, this increase being more marked than that found in normal mice. The increased concentration of

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vitamin A in the livers of castrated mice was unaffected by sex, gain or loss of weight, or by the addition of subcutaneously placed pellets of estrone.

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THE METABOLISM OF INTRAVENOUS TESTOSTERONE BY EXPERIMENTAL ANIMALS[1,2]

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ALTHOUGH it has never been isolated from blood, testosterone is usually considered to be the circulating androgen secreted by the testis. With the demonstration by Bischoff (1948) of the high solubility of testosterone in serum albumin, it became possible to administer large doses of free testosterone dissolved in serum albumin intravenously and to study the fate, distribution, and excretion of circulating testosterone.

EXPERIMENTAL

Testosterone solutions for intravenous administration were prepared by a modification of the method of Bischoff (1948). An excess of crystalline testosterone was brought into equilibrium with serum by continuous shaking at 37° C. for 12 hours. To reduce bacterial contamination, penicillin and streptomycin were added to the solutions. After equilibration the excess undissolved testosterone was removed by filtration. Aliquots of the filtrate were assayed for their testosterone content. The concentration of testosterone in the blood serums used in this study ranged from 450 to 750 ug./ml.

The rabbit, dog, and rat were used as experimental animals. The solutions were administered intravenously in doses varying from 20.4 to 53.5 mg. testosterone. Blood samples were taken at various intervals after administration and analyzed for their steroid content. In some experiments the animals were sacrificed and samples of various tissues taken for steroid analyses. In those animals in which the effects of hepatectomy and nephrectomy were studied, the kidneys were excised and the liver was functionally removed by ligating its entire blood supply.

Analytical Methods

A. Free Steroids:

Each blood sample, amounting to approximately 20 ml., was hemolyzed by the addition of an equal amount of distilled water and extracted five times with 40 ml. of redis-

Received for publication March 31, 1951.

[1] This work was supported in part by research grants from the National Cancer Institute of the National Institutes of Health, Public Health Service, from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and from Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

[2] The data in this paper were taken from a thesis presented by C. D. West to the Graduate School of the University of Utah in partial fulfillment of the requirements for the Ph.D. degree.

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tilled ether. Aliquots of tissues were homogenized, diluted to approximately 40 ml. in 100 ml. centrifuge tubes, and extracted in the same way as the hemolyzed blood. The dried ether extracts were partitioned between hexane and 70% ethanol. The 70% ethanol fraction was then dried, taken up in hexane, and chromatographed on aluminum oxide as described by Samuels (1947). The absorption maximum at 240 mμ. was used for determination of the α,β unsaturated ketones in the fractions. 17-Ketosteroids were determined by the Callow modification of the Zimmermann reaction (Callow, Callow and Emmens, 1938).

To test the accuracy and sensitivity of the analytical procedure model experiments were run in which testosterone or androsterone were added to blood or tissue. As little as 10 μg. of testosterone was recovered with an accuracy of 100 ± 10%. With 40 μg. the maximum error was ± 5%. Since fatty tissues presented the greatest problem in differential extraction, 576 μg. of testosterone were added to 10 g. of fat and analyzed as described. In duplicate samples the recoveries were 94.5% and 91.6%. In a similar manner it was demonstrated that as little as 5 μg. of added androsterone could be identified in 20 ml. blood, and that 25 μg. could be recovered to the extent of 82 ± 8%.

B. "Conjugated" Steroids:

Steroid sulphates or glucuronides would not be extracted by the procedure outlined above. The aqueous solutions after the ether extraction were therefore diluted to 100–300 ml., depending on the amount of blood or tissue involved, and 10% by volume of concentrated hydrochloric acid was added. The acid mixture was boiled for 15 minutes under reflux and thoroughly extracted with ether. The ether solutions were washed with 2.5 N NaOH, 8 N H_2SO_4, and then with water until neutral. It was also necessary to give the blood extracts an additional wash with saturated $NaHCO_3$ solution to remove all of the pigment. The dried extracts were then carried through the partitioning and chromatography previously described.

To test the hydrolytic procedure, 2 μmols of sodium androsterone sulfate were added to each of 4 samples of 20 ml. blood and carried through the above procedure. All recoveries were between 51% and 52.5% of the theoretical. The consistent high loss was probably due to the technique of hydrolysis, as Venning has shown for urines (Venning, 1942). Since checks among quadruplicates were good, however, it was felt that the values could be used as gauges of relative values.

RESULTS

Disappearance of testosterone from the circulating blood:

The rate of disappearance of intravenous testosterone from the circulation of two intact dogs and two intact rabbits is indicated in Figure 1. In this figure the percentage of the "original blood level" of testosterone is plotted against time. The "original blood level" is that concentration of testosterone to be expected in the blood if it did not disappear from the circulation. By estimating the blood volume from the total body weight and knowing the amount of testosterone administered, this theoretical concentration of testosterone in the blood can be closely approximated.

It is apparent that testosterone administered intravenously disappeared very rapidly from the blood. In all four animals 90% or more of the testos-

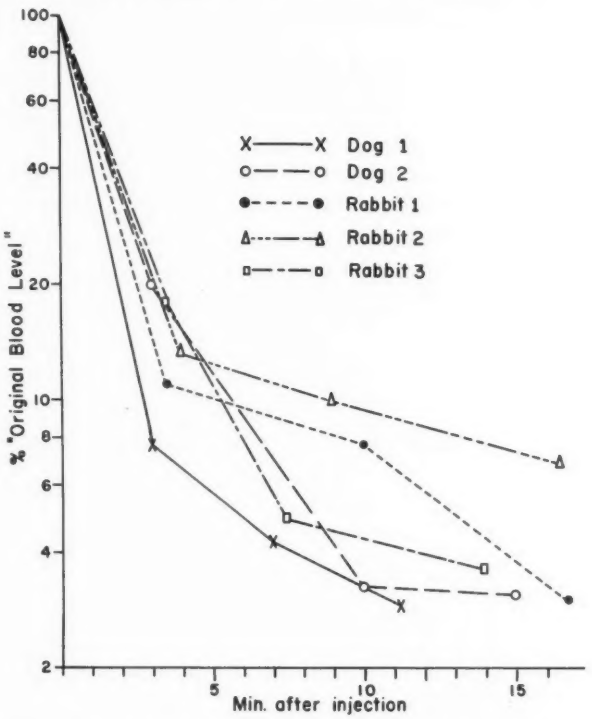

FIG. 1. Change in levels of testosterone in the blood of dogs and rabbits after the intravenous injection of the hormone dissolved in homologous serum. "Original blood level" is that concentration which would have resulted if the testosterone injected were distributed throughout the estimated blood volume.

terone had left the circulation within 10 minutes after administration. The process appears to be biphasic, the remaining 10% disappearing at a much slower rate relative to concentration.

Effect of the liver and kidney:

Previous investigators have demonstrated by *in vitro* experiments that the liver and kidney metabolize testosterone (Samuels, McCaulay, and Sellers, 1947; Clark and Kochakian, 1947; West and Samuels, 1949). To determine what role these organs might play in the disappearance of testosterone from the circulation, testosterone was injected into an intact anesthetized animal and periodic blood samples analyzed for testosterone. The same animal was then hepatectomized, testosterone again administered, and blood samples again taken periodically. The procedure was

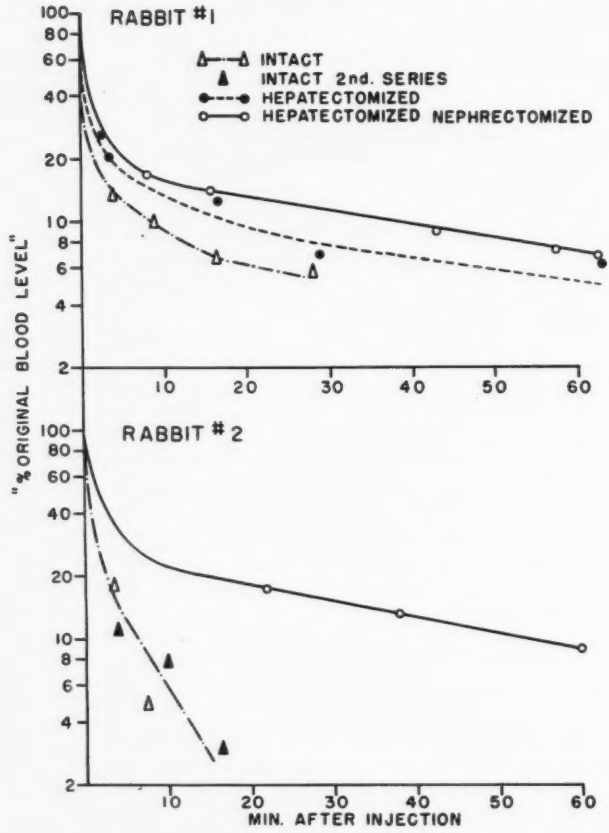

FIG. 2. Effect of hepatectomy and nephrectomy on the levels of testosterone in the blood after the intravenous injection of the hormone dissolved in serum.

repeated for a third time after the same animal had also been nephrectomized.

Experiments of this type were done in three dogs and two rabbits. The results in two typical experiments are indicated in Figure 2. Neither removal of the liver alone nor the elimination of the kidneys in addition affected the rapid rate at which the testosterone disappeared during the first phase. During the second phase, however, the elimination of each organ decreased the rate of disappearance.

Concentration of testosterone in tissues:

In all the experiments, 75% or more of the testosterone had disappeared from the circulation within ten minutes after injection, even in a hepatec-

tomized, nephrectomized animal. Obviously the liver and kidney were not involved in this loss and it became necessary to look for other explanations.

One possible explanation was that the testosterone had diffused out of the circulation into the tissues. To check this possibility, rabbits were injected intravenously with testosterone and sacrificed one hour after injection. Samples of various tissues were removed at that time and analyzed for their testosterone content. The results of these analyses are summarized in Table 1.

TABLE 1. TESTOSTERONE IN VARIOUS TISSUES FOLLOWING INTRAVENOUS ADMINISTRATION IN RABBITS

Tissue	Total tissue gms.	Concentration testosterone/100 gms.	% dose in tissue
Rabbit No. 1, 3.9 kgm., injected with 21.5 mgm. hormone:			
fat	364.0	1140	19.4
skeletal muscle	1720.0	204	16.3
testes	4.9	130	
heart muscle	9.3	350	0.2
lung	23.4	458	0.5
spleen	1.1	452	[illegible]
blood	258.0	448	5.9
kidney	10.1	370	0.2
liver	195.0	0	0
prostate	1.5	0	0
totals:	2587.3		42.5
Rabbit No. 2, 2.9 kgm., injected with 21.5 mgm. hormone:			
fat	270.0	2140	26.8
heart muscle	5.7	724	0.2
skeletal muscle	1320.0	222	13.7
blood	192.0	597	5.3
totals:	1787.7		46.0

All tissues tested, with the exception of liver and prostate, contained appreciable amounts of testosterone. The tissues, in order of decreasing concentration, were: fat, lung, spleen, kidney, heart muscle, skeletal muscle, and testis. Fat had by far the highest concentration. Identical tissues from normal non-injected male rabbits were analyzed simultaneously and were negative for α,β unsaturated ketosteroids. The testosterone was present in the tissues in the free form; it was readily extractable with ether, and hydrolysis did not yield additional testosterone.

There were no 17-ketosteroids by the Zimmermann reaction in the extracts of either unhydrolyzed or hydrolyzed tissues or blood. Neither α,β unsaturated ketosteroids nor 17-ketosteroids could be demonstrated in the urine passed by these animals during the experimental period.

An attempt was made to determine the proportion of the total amount of testosterone administered which was present in the various tissues studied (Table 1). This was calculated from the testosterone concentration

in the given tissue and the total weight of the tissue in the whole animal. In the case of organs such as heart, spleen, liver, kidney, etc., the tissue weights were obtained directly; in the case of such tissues as fat, skeletal muscle, and blood, direct weighing was impossible, and certain approximations had to be made from data on body composition in the literature.

In Rabbit No. 1 42.5% of the administered testosterone could be accounted for in an analysis of tissues making up 67% of the total body weight. In Rabbit No. 2, an hepatectomized, nephrectomized animal, only 46% of the injected testosterone was accounted for in tissues making up 65.5% of the hepatectomized, nephrectomized carcass weight (Table 1). These tissues accounted for 41.7% of the hormone in the intact rabbit. It does not seem, therefore, that removal of the liver and kidneys greatly affected the distribution of testosterone among the tissues during the first hour after injection.

To determine how long testosterone stayed in the fatty tissues after intravenous injection, the following experiment was run. Eighteen rats were divided into three groups of six rats each. Rats in Group 1 served as controls; each received 0.6 ml. serum intravenously. The rats in Group 2 were each injected with 0.822 mg. of testosterone dissolved in 0.6 ml. of serum. These groups were sacrificed one hour after injection. Rats in Group 3 received the same treatment as those in Group 2, except that they were sacrificed three hours after injection. Samples of fat were taken from each rat and pooled within groups for determination of testosterone.

The samples of fat from the control (Group 1) contained no demonstrable testosterone. There was a concentration of 913 μg. testosterone per 100 gm. fat in the animals sacrificed one hour after injection with the steroid (Group 2), but no measurable amount in the fat of those rats sacrificed three hours after injection (Group 3). Thus it would appear that testosterone stays in the fat stores for only a short period of time after intravenous administration.

DISCUSSION

From these data the sequence of events in the fate of intravenous testosterone seems first to involve rapid diffusion from the circulation into the tissues, particularly the fat stores. This occurs during the first few minutes.

The mechanism of this early removal would appear to be twofold. The great concentration in fat is probably a function of the relative solubilities of the hormone in it and in plasma. In these experiments, however, the concentrations in tissues other than the fat stores bore no relation to their relative fat content. Since the amounts present per unit volume of water greatly exceeded the solubility of testosterone in physiological salt solutions, it would seem probable that protein binding similar to that with

serum albumin was involved. This may be the fraction which is associated with the anabolic action observed so soon after intravenous injection of testosterone-albumin solutions in humans (West, Tyler, Brown and Samuels, 1951).

The fact that all the testosterone which was found in the tissues after injection was readily extracted with ether before hydrolysis indicates that the chemical bonding between the tissues and the testosterone which was recovered was weak. Living tissue, however, may also combine with testosterone in such a way that the hormone is destroyed during the hydrolytic process. Even in the nephrectomized, hepatectomized animal, less than half the injected hormone was recovered in tissues representing a large part of the animal's mass. The possibility of such destruction can be ruled out only be use of a variety of hydrolytic procedures.

The data showing the decreased rate of disappearance of testosterone from the circulation of hepatectomized animals indicate that the liver is a site of metabolism in the whole animal. Nephrectomy had an additional effect in the hepatectomized animal. It would seem, therefore, that *in vivo*, as *in vitro*, both orgnas play a part in the metabolic action on the testosterone molecule.

SUMMARY

The fate of testosterone administered intravenously in experimental animals has been investigated. The injected hormone diffused rapidly from the circulation into various tissues of the body, the highest concentrations being found in fat. The testosterone apparently did not stay in the fatty tissues for any great length of time since within three hours no measurable amounts remained.

Evidence is presented to indicate that the liver and kidney are both sites of testosterone metabolism in the mammal. That other sites than liver and kidney might be involved in the metabolism of testosterone was indicated by studies in hepatectomized, nephrectomized animals in which less than 50% of the testosterone injected could be accounted for in a major proportion of the tissues.

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EFFECT OF SITE OF INSEMINATION UPON THE FERTILITY OF GONADOTROPHIN-TREATED RABBITS OF DIFFERENT REPRODUCTIVE STAGES[1]

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THAT the fertilizability of artificially ovulated eggs is dependent in part upon the stage of the reproductive cycle in which ovulation is induced has been demonstrated in cattle (Casida *et al.*, 1940, 1943), sheep (Murphree *et al.*, 1944), rabbits (Boyarsky *et al.*, 1947; Murphree *et al.*, 1947; Austin, 1949) and swine (Tanabe *et al.*, 1949). Induction of ovulation in females by use of gonadotrophic hormones when their ovaries contain functional corpora lutea has yielded few or no fertilized eggs with either natural mating or artificial insemination, whereas treatment in the follicular phase has resulted in good fertility.

An association has been found between the age of the corpora lutea in the rabbit and the degree of suppression of fertility (Boyarsky *et al.*, 1947; Austin, 1949). The corpus luteum hormone, progesterone, has been demonstrated as one endocrine entity concerned in the suppression of fertility in the experimentally ovulated ova (Boyarsky *et al.*, 1947). The administration of estrogens to females having active corpora lutea in their ovaries shortly before the induction of ovulation has improved fertility slightly (Boyarsky *et al.*, 1947).

One possible explanation for the inhibitory influence of the corpora lutea upon fertility is that the sperm are adversely affected when deposited within the tract of the luteal-phase female. The rate of sperm transport in the reproductive tract of the pseudopregnant rabbit is not known, but if it is in any way depressed the sperm may not reach the fertilization site in sufficient numbers to effect fertilization within the fertilizable life period of the ovum. Austin (1949) postulated the failure of sperm to ascend the tubes in sufficient numbers and also the existence of conditions in the oviducts of pseudopregnant rabbits which would militate against fertilization, even when abundant sperm were placed directly in the oviduct. In the present work the number of fertilized ova resulting from induced ovulation

Received for publication May 4, 1951.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Paper No. 461 from the Department of Genetics.

has been studied when the sperm were deposited at different levels of the reproductive tract. In addition the number of artificially ovulated ova which were fertilized has been studied when near-minimal numbers of sperm were deposited in the uterus.

METHODS

Adult estrous, pseudopregnant, and pregnant rabbits from mixed breeding of medium weight stock were used in this study. Estrous females had been caged individually at least 30 days and showed a willingness to accept the male. Pseudopregnant females were obtained by mating estrous does to vasectomized males 8–10 days before the experimental insemination; pregnant females were obtained by mating estrous does to known-fertile males 10 days before insemination. One control group of estrous females was ovulated by mating to vasectomized males. All other groups were treated at the time of artificial insemination by intravenous injection of either one gram equivalent of an unfractionated sheep pituitary extract ("B" fraction of McShan and Meyer, 1943) or 100 I.U. of chorionic gonadotrophin to induce ovulation.

Semen used for insemination was collected with the artificial vagina from males of proven fertility and diluted at the rate of one part of semen to four parts of modified Krebs solution (Lardy and Phillips, 1943). The control group of estrous animals was inseminated by passing a glass insemination tube through the vulva into the anterior part of the vagina. The other groups were inseminated by exposing the reproductive tract through a midventral laparotomy with the animal under light anesthesia and by injection of semen with a hypodermic needle into the lumen of the vagina or uterus. In vaginal insemination 0.5 cc. of diluted semen was deposited by puncture of the anterior vaginal wall. In uterine insemination 0.25 cc. of diluted semen was deposited in each horn, either by puncture of the uterine wall at the anterior or posterior end or by puncture of the vaginal wall and insertion of the needle through the cervical canal into the uterus (cervico-uterine). The anterior uterine site alone was used in the pregnant group in which semen was deposited in the uterus. The numbers of sperm used in vaginal and uterine inseminations were consequently approximately equal. All animals were sacrificed 30–48 hours after the experimental insemination, the oviducts removed and flushed with modified Krebs solution to recover the ova. The percentage of fertilized (i.e., equally-cleaved) ova was obtained by examination under the binocular microscope.

RESULTS

No differences were apparent in fertilization rates obtained by the three methods used for uterine insemination; anterior, posterior, and cervico-uterine. The data have therefore been pooled (Table 1). The 14 estrous animals of the control group yielded 149 ova of which 98.7% were fertilized. Five estrous does inseminated through the vagina and nine through the uterus gave comparable fertilization rates of 97.3 and 94.7% respectively. However, in the 15 pseudopregnant females which were inseminated through the vagina, the rate was only 3.1%. This marked influence of pseudopregnancy on fertility was considerably lessened when the semen was deposited in the uterus. The value of 61.0% obtained from 17 animals, however, was still significantly lower than the comparable figure of 94.7% obtained in estrous rabbits inseminated through the uterus.

A somewhat similar situation was realized in the two 10-day pregnant groups. The fertilization rate resulting from vaginal insemination of six does was 12.1%, one female having all four of the ova recovered from her fertilized. Again uterine insemination increased the fertilization rate, in this case to 83.3% for four females. This, however, was not significantly different from the group of estrous doses inseminated through the uterus.

The pronounced reduction in fertility in the pseudopregnant rabbit, previously demonstrated by Boyarsky *et al.* (1947), Murphree *et al.* (1947), and Austin (1949) has been confirmed. The improvement in fertilization rate obtained by deposition of semen in the uterus indicates that diffi-

TABLE 1. EFFECT OF INSEMINATION SITE ON FERTILIZATION RATE IN ESTROUS, PSEUDOPREGNANT, AND PREGNANT RABBITS

Reproductive stage	Site of insemination	No. of females	Fertilized ova	Unfertilized ova	Fertilization %
Estrous	Vagina[1]	14	147	2	98.7
Estrous	Vagina[2]	5	36	1	97.3
Estrous	Uterus[2]	9	54	3	94.7
Pseudopregnant	Vagina[2]	15	4	126	3.1
Pseudopregnant	Uterus[2]	17	61	39	61.0
Pregnant	Vagina[2]	6	4	29	12.1
Pregnant	Uterus[2]	4	20	4	83.3

[1] Ovulation induced by mating to vasectomized males; semen deposited in anterior vagina with glass tube passed through vulva.
[2] Ovulation induced by intravenous injection of gonadotrophin; insemination by mid-ventral laparotomy.

culties in sperm transport through the cervix may account for a considerable portion of the infertility found in those animals in whose ovaries active corpora lutea are present. Fertility in the groups of pregnant and pseudopregnant animals inseminated through the uterus was extremely variable. Three of the total of 21 females had no fertilized ova, nine showed partial fertility, and nine had all ova fertilized, This variability is so great as to indicate a possible differential from one female to another either in the fertilizability of the ova or in the success of sperm transport.

A test of the fertilization rate was next made in estrous and pseudopregnant females with uterine insemination and using numbers of sperm only slightly above the minimum necessary for fertilization in estrous rabbits (Cheng and Casida, 1948). It was anticipated that this test would accentuate the difference between the estrous and pseudopregnant rabbit if the problem in the pseudopregnant animal were at least partially one of sperm transport. Semen was collected and sperm counts made; only those ejaculates which would contain between 100,000 and 200,000 sperm per 0.5 cc. of diluted semen were used for insemination purposes. The semen

was diluted with modified Krebs solution in the proportion of one part of semen to 999 parts of diluent. Ovulation was induced in three estrous and nine pseudopregnant females and they were inseminated in the anterior end of each uterine horn with 0.25 cc. of the diluted semen. The results (Table 2) were very similar to those obtained with vaginal insemination in estrous and pseudopregnant rabbits (Table 1). From the estrous does 13 of 15 eggs were fertilized, and from the pseudopregnant rabbits only three of 59 ova were fertilized. These results appeared to suggest that too few sperm were present in the oviducts of the pseudopregnant female to fertilize more than an occasional ovum.

TABLE 2. EFFECT OF MINIMAL NUMBERS OF SPERM ON FERTILITY IN ESTROUS AND PSEUDOPREGNANT RABBITS

Stage	No. of sperm insem. in 1000's	No. of females	Fertilized ova	Unfertilized ova	Fertilization %
Estrous	116	1	3	1	
	154	1	4	1	
	168	1	6	0	
		3	13	2	86.6
Pseudopregnant	116	4	2	19	
	154	3	1	27	
	168	2	0	10	
		9	3	56	5.1

Observations on uterine contents

One feature of interest in this study was the incidence of endometritis with accompanying pyometra in the uteri of the pseudopregnant and pregnant rabbits. In the uteri examined for this condition, none of 10 estrous females inseminated through the vagina or uterus showed this condition, while three of 14 uteri from animals inseminated through the vagina and 11 of 15 uteri from animals inseminated through the uterus during the luteal phase of the reproductive cycle contained appreciable amounts of pus. This condition appears to be restricted to uteri (especially those in which semen was deposited into uteri) which have been under the influence of active corpora lutea; the condition further appears to develop rapidly following insemination since such uteri are grossly normal at laparotomy two days previous to autopsy or less. The cause and nature of this reaction is at present unknown. A somewhat similar condition was noted in the sows treated with gonadotrophin during the luteal phase studied by Tanabe *et al.* (1949).

At the time of autopsy 30–48 hours after re-ovulation and insemination in the ten pregnant females used in this study, it was found that the original

embryonic sites were undergoing resorption. It had already progressed considerably as indicated by gross measurements and by the condition of the contents of the sites. As a further check six 10-day pregnant does were given an injection of gonadotrophin without insemination and their embryonic sites compared 30 hours later with those of normal 11- and 12-day pregnancies. Again resorption was far advanced in the treated animals. The corpora lutea of normal pregnancy were highly vascularized with prominent ovulation papillae. The original corpora in re-ovulated pregnant females, on the other hand, were pale in color and lacked prominent papillae; no significant difference in size from the normal was found. The intravenous administration of a large dose of gonadotrophin appears to have initiated involutionary changes in the corpora present at the time of injection. These regressive changes must begin very soon after the injection in order to be detected 30 hours later. The regression noted in the embryonic sites at autopsy may be dependent in part on the secretory failure of these corpora lutea.

Recently Burdick and Crump (1951) have produced ovulation in pregnant mice without interfering with the normal course of the pregnancy. This suggests a species difference in response assuming other elements of the treatment to be comparable to that given the rabbit.

DISCUSSION

The observation, in the experiments described here, that the fertilization rate in pseudopregnant rabbits treated with gonadotrophin was markedly improved by deposition of the semen above the cervix suggests that the initially-observed infertility was in large part due to difficulties in sperm transport. Further evidence in support of this theory is found in the greater effect on the pseudopregnant rabbit than on the estrous of decreasing the number of sperm used in uterine insemination.

Austin (1949) suggested that the apparent infertility following vaginal insemination might be due simply to the low order of probability that any of the greatly reduced number of sperm gaining the oviducts would meet and fertilize an egg. After he introduced sperm into the oviducts of estrous and pseudopregnant females and found 14 fertilized in a total of 19 ova as compared to four fertilized in a total of 18 ova respectively, he found it necessary to modify his first suggestion and to postulate the existence of a set of conditions in the oviduct of the pseudopregnant rabbit which would interfere with the fertilization. He did not mention the possibility that irritation of the mucosa itself consequent to handling and injection of semen into the oviduct may have interfered. He rejected the suggestion of Murphree *et al.* (1947) that eggs matured and ovulated during the luteal phase may be inherently defective on the basis that it is most unlikely that only a portion of the ova would be so affected.

Boyarsky (1947) found no evidence of fragmentation up to 45 hours in unfertilized eggs obtained from estrous females mated to vasectomized males. But in estrous and pseudopregnant rabbits in which ovulation was hormonally induced, 2.6 and 4.4%, respectively, of the ova had begun to fragment within the same period of time. This suggests that the artificial maturation and induced ovulation of these ova had some injurious effect upon them. Induction of ovulation in pseudopregnant does through administration of exogenous gonadotrophins may cause follicles in various stages of maturation (whose terminal growth is normally suppressed by the presence of progesterone) to undergo the final stages of development in an accelerated manner. Such artificially ripened ova may be of inherently different ages and resultant viability. In view of this it seems not at all illogical to postulate that only a portion of the ova may be inherently defective and consequently that animals in the luteal phase of the reproductive cycle show variable and partial fertility.

One method for attacking this problem would be to transplant freshly ovulated ova from estrous and pseudopregnant females into the oviducts of previously inseminated estrous does. If the proportion of ova obtained from pseudopregnant rabbits which were fertilized after being placed in the new environment (presumably normal for sperm transport) remained at a lower level than for ova from estrous does so treated, the indication would be that some of the ova from the pseudopregnant were inherently infertile. If no difference was found, on the other hand, the conclusion could be drawn that conditions for fertilization within the pseudopregnant oviduct are to blame.

SUMMARY

Estrous, 10-day pseudopregnant, and 10-day pregnant rabbits were treated with gonadotrophins to induce ovulation, and were inseminated by exposing the reproductive tract through midventral laparotomy and injection of semen into the vaginal or uterine lumen. The animals were sacrificed 30–48 hours later to determine the fertilization rate.

Site of insemination had little effect on the high fertilization rate in estrous animals. Vaginal inseminations of pseudopregnant and pregnant does gave very poor fertility, while uterine insemination materially improved fertilization.

Estrous and pseudopregnant rabbits were artificially ovulated and inseminated through the uterus with low numbers of sperm. The fertilization rate in the pseudopregnant animals was markedly lower than in the estrous.

It is concluded that some interference with the transport of sperm through the cervix accounts for much of the infertility in luteal-phase rabbits.

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HISTAMINE TOLERANCE IN RATS WITH INTRAMUSCULAR ADRENOCORTICAL TRANSPLANTS

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PROGRESSIVE resumption of function of adrenocortical transplants to the dorsal musculature in rats has been followed by submitting the animals to various stresses, such as histamine poisoning, exposure to cold, and water intoxication (Komrad and Wyman 1950). Seven days after operation rats with transplants recovered from doses of histamine fatal to adrenalectomized animals, but the maximum efficiency of the transplants was not tested. Although it has long been known that adrenalectomized rats are less resistant to histamine than normal rats (Crivellari 1927), and that rats with adrenocortical transplants can withstand doses of histamine which would kill adrenalectomized animals (Ingle 1937; Wyman and tum Suden 1937), we have found no report of a systematic quantitative determination of the degree of protection provided by transplants against histamine poisoning. Moreover the quantitation in previous reports is in terms of the m.l.d., whereas calculation of the LD_{50} is believed to be a more precise indication of pharmacologic toxicity. A study was made, therefore, of the resumption of function of intramuscular transplants by calculation of the LD_{50} of histamine at various intervals after operation.

METHODS

A total of 289 male albino rats of the Wistar strain were adrenalectomized by the dorsal route under ether anesthesia. In 249 rats the glands were trimmed of fat, bisected, and autotransplanted to the dorsolumbar and spinotrapezius muscles. These animals were given saline drinking water for three days, then tap water, and the remaining 40 adrenalectomized animals were given saline water for five postoperative days. Purina Laboratory Chow was fed *ad libitum* to all animals.

Histamine phosphate (Burroughs Welcome), one milligram of crystals containing 0.362 mg. of histamine base, was injected intraperitoneally in solutions prepared so that 0.5 ml. per 100 gm. of body weight was given. At the time of the test the mean weight of the rats was 130 ± 30 gm. Each of three doses of histamine selected so that the responses obtained were within a 16 to 84 per cent frequency was injected into 7 to 12 rats on a given day. Any animal not dead four hours after the injection was considered to have recovered. The LD_{50} was determined by the method of Litchfield and Wilcoxon (1949). Forty sham operated and 40 adrenalectomized controls were tested simultaneously with the experimental rats.

Received for publication May 21, 1951.

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RESULTS AND DISCUSSION

The data in Table 1 indicate that intramuscular adrenocortical transplants afford a statistically significant progressive increase in protection against histamine poisoning as measured by the LD_{50} up to 10 days after operation, but no further increase in protection from the tenth day onward. These results are in accord with earlier findings (Komrad and Wyman 1950) which disclosed a progressive resumption of function up to the tenth day when a normal response to cold and considerable protection against water intoxication were obtained. Apparently, ten day old adrenal

TABLE 1. LD_{50} OF HISTAMINE IN RATS WITH INTRAMUSCULAR ADRENOCORTICAL TRANSPLANTS

	Adrenal-ectomized	Transplants—days after operation						
		5	7	10	13	17	20	30
LD_{50} of histamine and limits of error mg./100 gm.	5.8 (3.9–8.7)	10.2 (7.7–13.7)	16.5 (12.2–22.2)	27.0 (22.8–31.8)	26.0 (20.0–33.8)	27.0 (24.7–29.9)	31.0 (25.8–37.1)	28.0 (22.9–34.1)

transplants can secrete not only enough hormone to meet the animal's "resting" needs, but even the additional amounts required under severe stress. A completely normal response to histamine administration, however, was not attained. Although a determination of the LD_{50} for normal rats was not made, it is considerably higher than that of animals with one month old transplants. The LD_{50} of the latter is about 28 mg. per 100 gm. body weight, and ten normal rats injected with 40 mg. per 100 gm. survived. Marmorston-Gottesman and Gottesman (1928) found that 160 mg. of histamine phosphate per 100 gm. were required to reach the m.l.d. for normal rats. Whether this discrepancy is the result of inefficiency of the transplants or medullary deficiency is not yet known, but it seems likely that under severe stress both are involved. Although Gordon (1950) has recently concluded that the adrenal medulla is not essential for the liberation of ACTH from the pituitary following stress, which is in agreement with our findings, the work of Gershberg *et al.* (1950) has shown that epinephrine plays some rôle in ACTH release. It is not surprising, therefore, that demedullation deprives the animal of part of its mechanism for maintaining homeostasis under conditions of stress. Ingle (1937) and Wyman and tum Suden (1937) likewise concluded that both the adrenal medulla and cortex are necessary for maximum protection against histamine intoxication. However, it is highly probable that the deficiency in medullary hormone is not the only factor responsible for the inefficiency of adrenocortical transplants under conditions of general body stress. Since the cortical tissue is in a different anatomical position, the arterial blood supply and hence the nutrition is altered in source and perhaps in quantity. The

slight differences in temperature may decrease the rate of hormonal production and the alteration in venous distribution may interfere with the metabolism of the secreted hormone.

The technique of transplantation of the adrenal gland has been used to eliminate the medulla in order to study cortical activity uncomplicated by medullary secretion. The enucleation technique described by Evans (1936) is better for this purpose, however, since it is quicker and has a greater chance of success. The main value of the transplantation technique is that the cortex may be placed on any organ where it will form anastomoses with the vessels of that organ so that its hormone may be delivered directly to it without passing through the steroid inactivating areas of the body.

SUMMARY

Adrenocortical transplants to the dorsal musculature of rats progressively resume their ability to protect the animal against histamine poisoning as measured by the LD_{50} technique. The results, together with those from earlier investigations, indicate that by the tenth day after operation the transplanted cortical tissue is capable of secreting sufficient hormone to meet the severe requirements of an acute stress in addition to the daily needs of the animal.

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"PANENDOCRINECTOMY" AND THE SERUM PROTEINS IN THE DOG

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IN THIS investigation we studied the effect of multiple extirpation of endocrine glands on the serum proteins. It is known that following adrenalectomy in the dog (Remington *et al.*, 1941; Hartman *et al.*, 1942), cat (Levin, Leathem and Crafts, 1942) and rat (Leathem, 1945) there is a decrease in serum albumin and an elevation of serum globulin concentration. The rise in globulin is not sufficient to maintain normal total protein levels. The hypophysectomized animal also exhibits lowered serum albumin values (Goldberg, 1938; Levin and Leathem, 1942; Smith and Levin, 1942). Results are not in complete accord as to whether total protein values are normal or slightly below normal after hypophysectomy.

Diseases which effect the endocrine system tend to decrease serum concentrations of albumin and total protein. In Addison's disease serum albumin is low (McCullagh and Lewis, 1945) and the concentration of this protein is increased, though not to normal, when desoxycorticosterone acetate or adrenal cortical extract or cortisone or a combination thereof is administered. The total protein is within range of normal. Cushing's syndrome has been shown electrophoretically to also have depressed plasma albumin concentration with normal protein values (Lewis and McCullagh, 1947). Hyperthyroidism in the human is characterized by decreased plasma albumin concentration. Alpha globulins are elevated. These values are restored to normal following thyroidectomy. Hypothyroidism exhibits a low level of alpha globulin, an increase in beta globulin but with a depression of plasma albumin (Lewis and McCullagh, 1944; Hooft and van Belle, 1944). Lewis and coworkers (1944) have shown also that though the severe uncomplicated case of diabetes mellitus has a normal total protein, serum albumin is depressed while beta globulins are quite elevated. These protein abnormalities are not observed in the mild cases of diabetes.

This present investigation was undertaken to study further the effect of the endocrine system on serum proteins in the dog following adrenalectomy, hypophysectomy and a combination of both procedures. In addition serum proteins were studied in the "panendocrinectomized" dog. "Panendocrinectomy" is defined as extirpation of adrenals, pituitary, pancreas, testes (or ovaries), thyroid and parathyroid glands.

Received for publication May 22, 1951.

METHODS

All experiments were carried out on healthy male and female mongrel dogs. Adrenalectomy was performed in two stages and the animals were maintained on one milligram of desoxycorticosterone acetate intramuscularly and two grams of salt added to the drinking water each day. The technique of Dandy and Reichert (1925) was followed for hypophysectomy. No supportive therapy was necessary in thdse animals. Two dogs survived "panendocrinectomy." The order of glandular extirpation is as mentioned above. These particular dogs developed complete anorexia necessitating feeding through a gastric cannula. The diet consisted of 50 calories per kilogram to which was added two grams of salt, calcium lactate, choline dihydrogen citrate and vitamins. Though usually requiring more adrenal cortical replacement one dog was maintained on two milligrams of desoxycorticosterone daily for four weeks. During this period he appeared healthy and active. His original heavy coat was replaced by short, fine hair. Despite a gradual weight loss, to the casual observer he appeared to be a normal dog. In an animal so prepared minor electrolyte variations assumed grave proportion. Electrolyte imbalances resulted in death within 48–72 hours unless cortisone and replacement therapy were administered.

Two adrenalectomized dogs were treated with cortisone. One (#53) received 100 mg. intramuscularly each day for seven days. Another dog (#774) received only 50 mg. each day for a period of four days. Hypophysectomized dog number 13 was treated with adrenocorticotrophic hormone, 50 mg. intramuscularly each day, for five days.

Total protein was determined by the micro-Kjeldahl method of Ma and Zuazaga (1942). Serum albumin values were obtained according to the procedure of Howe (1921) as modified by Weichselbaum (1946).

RESULTS

The results obtained were more significant when preoperative and postoperative values were applied to each dog individually rather than comparing a mean preoperative value of all dogs in the series to total mean postoperative values (Table 1).

1. Four dogs were adrenalectomized. One dog developed an increased serum total protein concentration of 5.8 per cent (6.37 to 6.74 gm./100 ml.) while the rest had a mean decrease of 7 per cent below normal. This same animal demonstrated a marked decrease in serum albumin concentration

TABLE 1. MEAN SERUM PROTEIN VALUES FOLLOWING ENDOCRINECTOMY IN THE DOG

	Adrenalectomy				Hypophysectomy					Adrenalectomy and Hypophysectomy		"Pan-endocrinectomy"	
Dog number	16	53	735	774	13	143	206	213	258	53	13	258	53
	Total protein grams per 100 ml.												
Preoperative	6.27 ±0.30	6.71 ±0.34	6.02 ±0.32	6.37 ±0.26	5.88 ±0.12	6.74 ±0.42	6.22 ±0.11	5.80 ±0.03	6.87 ±0.3	6.71 ±0.34	5.88 ±0.12	6.87 ±0.30	6.71 ±0.34
Postoperative	6.18 ±0.33	6.07 ±0.38	5.41 ±0.64	6.74 ±0.73	6.05 ±0.4	6.57 ±0.48	6.16 ±0.28	5.45 ±0.26	6.78 ±0.23	5.59 —	5.50 ±0.23	4.68 ±0.29	6.12 ±0.11
	Albumin grams per 100 ml.												
Preoperative	3.92 ±0.22	3.72 ±0.04	3.48 ±0.17	3.95 ±0.21	3.40 ±0.12	3.76 ±0.3	4.29 ±0.11	4.06 ±0.22	3.92 ±0.15	3.72 ±0.04	3.40 ±0.12	3.92 ±0.15	3.72 ±0.04
Postoperative	3.12 ±0.19	2.40 ±0.35	2.41 ±0.30	2.86 ±0.38	2.86 ±0.2	3.12 ±0.37	3.86 ±0.1	3.21 ±0.23	3.39 ±0.29	2.63 —	1.97 ±0.11	1.94 ±0.17	1.98 ±0.21

as did each of the others. The average depression amounted to 25.4 per cent of normal.

2. Five hypophysectomized dogs were studied and each had a decrease in serum albumin concentration. The mean fall was 22 per cent of normal. Total protein alterations were not consistently in the same direction. While one dog had a 2.9 per cent elevation of postoperative as compared to preoperative total protein values (5.88 to 6.05 gm./100 ml.) the other four had a mean depression below normal of 2.5 per cent.

3. Adrenalectomy-hypophysectomy combination on two dogs produced an unexpected drop in total protein concentration of 11.6 per cent. Serum albumin also fell to 35.7 per cent of normal, or less than a summation of the decrease observed in the separate procedures.

Results following "panendocrinectomy" were difficult to assay as an extremely narrow electrolyte and nutritional balance existed in an animal so prepared. Comparing preoperative values with postoperative determinations made during healthiest periods, the following data are obtained: The total protein dropped an average of 20.9 per cent in the two dogs studied. It should be stated that the animal in better control had only an 8.3 per cent depression below normal, i.e., 6.71 to 6.17 gm./100 ml. Both dogs had a pronounced decrease in serum albumin, 49.7 and 43.5 per cent.

The above results are summarized in Table 1.

DISCUSSION

From the results observed it may be concluded that a definite endocrine-serum protein relationship exists. Extirpation of the pituitary results in a marked fall in serum albumin concentration. Adrenalectomy produced an even more pronounced depression in serum albumin. That the decreased concentration of this protein fraction observed following hypophysectomy

TABLE 2. MEAN SERUM PROTEIN VALUES FOLLOWING CORTISONE AND ACTH THERAPY IN THE ENDOCRINECTOMIZED DOG

	Adrenalectomy		Hypophysectomy
Dog Number	53	774	13
	Total Protein Grams per 100 ml.		
Preoperative	6.71	6.37	5.88
Postoperative	6.07	7.74	6.05
After Cortisone	7.64	8.58	
After ACTH	—	—	6.67
	Albumin grams per 100 ml.		
Preoperative	3.72	3.95	3.40
Postoperative	2.40	2.86	2.86
After Cortisone	3.68	3.33	
After ACTH	—	—	3.08

may not be due to secondary adrenal cortical hypofunction has been indicated by Levin and Leathem (1942). In their hypophysectomized rats treated with adrenal cortical extract or desoxycorticosterone acetate there was incomplete restoration of serum albumin values to normal. Furthermore, adrenocorticotrophic hormone as used in the present study also failed to return completely the serum albumin to pre-operative level in the hypophysectomized animal (Table 2). The depression of serum albumin concentration following adrenalectomy in the dog may be partially recovered following replacement therapy with salt, desoxycorticosterone, adrenal cortical extract or a combination thereof. With cortisone however the serum albumin level is significantly restored: 2.40 to 3.68 gm./100 ml. in one dog and 2.86 to 3.33 gm./100 ml. in the other animal studied. Cortisone further caused the total protein to be elevated above normal (Table 2). This appeared to be a simple reflection of the rise in albumin fraction. Removal of adrenals and pituitary produced a greater fall in albumin concentration than either procedure alone. "Panendocrinectomy," as described, results in albumin valued approximately 50 per cent of normal.

It is appreciated that serum albumin determination by Howe fractionation is not as accurate as that obtained by electrophoresis. Other investigators (Li, 1944; Moore *et al.*, 1944; Li and Reinhardt, 1947) of adrenalectomized and hypophysectomized animals using electrophoretic protein analyses have also demonstrated depressed serum albumin concentrations.

Alterations of total protein concentrations after endocrinectomy are not as striking as those of serum albumin. Following hypophysectomy there is no significant over-all change. Maintenance of normal or near normal total protein values despite a depression of serum albumin in hypophysectomized animals is due to decreased thyroid activity producing elevation of serum globulins (Moore *et al.*, 1944). There was an insignificant depression of total protein in the adrenalectomized dog. Adrenalectomy-hypophysectomy combination resulted in a total protein depression far greater than expected considering total protein variations following the separate procedures. Following "panendocrinectomy" this marked decrease in total protein was still observed.

It is difficult to determine whether the endocrine-serum protein relationship is a direct or an indirect mechanism. The effect of glandular extirpation on protein intake, absorption and excretion must be evaluated. It has been demonstrated that adrenalectomized rats treated with salt have low gastric secretion, and lowered acid and enzyme content (Tuerkischer and Wertheimer, 1945). Adrenalectomized rats are also known to be in a state of negative nitrogen balance if no replacement therapy as adrenal

cortical extract or desoxycorticosterone acetate is administered (Ingle and Oberle, 1946). Bennett (1936) has shown that following hypophysectomy there is decreased appetite and gastrointestinal absorption. Additional studies of Levin and Leathem (1943) on normal rats starved to 20 per cent weight loss reveal a change in protein concentrations in the same direction as that following hypophysectomy but the fall is of much less magnitude. In conclusion, decreased appetite, decreased protein intake and impaired absorption can occur following adrenalectomy, hypophysectomy, a combination of both procedures or "panendocrinectomy." These factors apparently only partially account for the marked depressions in the serum albumin and total protein observed.

CONCLUSIONS

1. Following hypophysectomy, bilateral adrenalectomy, a combination of both procedures and "panenocrinectomy" in the dog there is a decrease in serum proteins. The magnitude of this decrease occurs in the order listed. Depression in albumin concentration exceeds that of total protein.

2. Cortisone will completely restore serum protein values to normal following adrenalectomy in the dog. Adrenocorticotrophic hormone fails to elevate completely the depressed protein concentrations in the hypophysectomized animal.

3. Dogs will survive "panendocrinectomy" (extirpation of adrenals, pituitary, pancreas, testes or ovaries, thyroid and parathyroids) if adequate replacement therapy is administered.

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ENDOCRINE INFLUENCES ON PROTEINURIA IN THE RAT: EFFECT OF HYPOPHYSECTOMY[1,2]

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ENDOCRINE influences on renal function have been the object of much attention in the past, but endocrine effects on the renal excretion of protein have only recently been studied. Pickering and Prinzmetal (1940) first noted increased proteinuria in the rabbit following the administration of the renal enzyme, renin. Addis, Barrett, Boyd, and Ureen (1949) reported the production of an intense, transient proteinuria in the normal rat by the intraperitoneal injection of renin. Addis and Boyd (1949) observed that this proteinuria response to renin is abolished by bilateral adrenalectomy. In the adrenalectomized rat, the administration of desoxycorticosterone acetate (DOCA), cortisone, or adrenal cortex extract will restore the proteinuria response to renin. In addition, adrenalectomy has been found to diminish both the spontaneous proteinuria found in normal male rats and the proteinuria which follows the intraperitoneal injection of serum albumin (Addis, Marmorston, Goodman, Sellers, and Smith, 1950). Goodman *et al.* (1951) have found a marked increase in renin induced proteinuria in rats that had received ACTH. Finally, gonadectomy and the administration of testosterone propionate have been shown to affect spontaneous proteinuria in the rat (Sellers *et al.*, 1950).

The experiments reported herein were designed to study the effect of hypophysectomy on renin induced proteinuria and on the spontaneous proteinuria of normal male rats. In addition, the use of hypophysectomized rats has enabled us to study the effects of adrenal steroids on proteinuria in the absence of the pituitary hormones.

Received for publication May 28, 1951.

[1] This work has been supported by grants from the United States Public Health Service and the American Foundation for High Blood Pressure.

[2] We are indebted to Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products Incorporated for the testosterone propionate, Dr. Edwin E. Hays, and Dr. Irby Bunding of Armour and Company for the ACTH and growth hormone preparation, and Dr. G. Babcock, Jr. of Schering Corporation for the DOCA.

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EXPERIMENTAL PROCEDURE

One hundred and nine rats of the Slonaker-Addis strain (Addis and Gray, 1950) were used in these experiments. Rats were 80–100 days of age at time of operation. Hypophysectomy was performed under ether anesthesia by the para-pharyngeal approach. All rats were checked for completeness of hypophysectomy at autopsy at the termination of the experiments by examination of the sella turcica under the dissecting microscope and by comparison of organ weights with those of normal rats. Incompletely hypophysectomized rats were eliminated from the experiments. All animals were maintained on the ordinary stock diet,[5] and allowed water ad libitum. The following treatment schedules were used:

Group I: Hypophysectomized rats were untreated.

Group II: Hypophysectomized rats were injected subcutaneously with desoxycorticosterone acetate (DOCA) in sesame oil 0.5 mg. daily from the day of operation to termination of the experiment twenty-four days later.

Group III: Hypophysectomized rats were injected subcutaneously with 11-dehydro-17-hydroxycorticosterone (cortisone) 2.0 mg. daily in saline suspension from the day of operation to termination of the experiment twenty-four days later.

Group IV: Hypophysectomized rats were injected subcutaneously with hog pituitary adrenocorticotropic hormone (ACTH) 2.3 mg. (Armour Standard LA-I-A equivalent) dissolved in 1 cc. 0.85% saline immediately before injection, twice daily from the day of operation to termination of the experiment twenty-four days later.

Group V: Hypophysectomized rats were injected with pituitary growth hormone 0.2 mg. in 0.5 cc. saline, thyroxin 0.005 mg. in 0.5 cc. saline, and testosterone propionate 2.5 mg. in sesame oil (subcutaneous injections at separate sites) from the day of operation to the termination of the experiment seventeen days later.

Urine was collected for sixty minutes immediately following the intraperitoneal injection of four Goldblatt dog units of hog renin[6] diluted in 4 cc. of 0.85% sodium chloride solution on the 10th, 17th, and 24th postoperative days. These animals had subsisted on a 15% glucose, 0.4% saline and 0.5% vitamin B complex solution the night prior to renin injection. The detailed technique of the urine collections has been described in a previous publication (Addis *et al.*, 1949). Determination of urine proteins was performed by a slight modification of Kingsley's biuret method (1939).

RESULTS

I. *Effect of hypophysectomy on renin proteinuria*

Intraperitoneal injection of four units of renin in 4 cc. of 0.85% sodium chloride solution in the normal male rat is followed by a proteinuria which has a mean value of 57.4 mg./hr. in the first hour following renin injection. Hypophysectomized rats, both male and female, are incapable of responding to renin with significant proteinuria (Table 1). The only therapy that was effective in restoring renin proteinuria in hypophysectomized animals was cortisone. ACTH, DOCA, and the combination of thyroxin, growth

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[5] The stock diet contains 17 per cent protein and 0.5 per cent NaCl.

[6] We are grateful to Drs. Harry Goldblatt, Erwin Haas, and Hildegard Lamfrom for supplying us with the renin used in this study.

TABLE 1. EFFECT OF HYPOPHYSECTOMY AND REPLACEMENT THERAPY ON RENIN PROTEINURIA

Group	Sex	Number of rats	Number of observations	Renin Proteinuria mg./hr.
Normal	Male	42	45	57.4
Hypophysectomy	Male & Female	34	42	0.9
Hypophysectomy + DOCA	Male	27	53	1.34
Hypophysectomy + Cortisone	Male	20	57	21.8
Hypophysectomy + ACTH	Male	12	29	2.19
Hypophysectomy + growth hormone, testosterone, and thyroxin	Male	9	17	0.6

hormone, and testosterone were all ineffective in raising the urinary protein content of renin treated hypophysectomized rats above that of non-renin treated hypophysectomized controls.

II. *Effect of hypophysectomy on spontaneous proteinuria in the male rat*

Normal male rats spontaneously excrete from two to five times more protein in their urine than normal female rats (Addis, 1931, and Sellers *et al.*, 1950) (Table 2). We are accustomed to refer to this spontaneous

TABLE 2. EFFECT OF HYPOPHYSECTOMY ON SPONTANEOUS PROTEINURIA IN THE RAT

Group	Sex	Number of rats	Number of observations	Proteinuria* mgm./hr./ 100 cm.²	Standard deviation
Normal	Male	97	129	0.18	±0.047
Normal	Female	34	65	0.10†	±0.058
Hypophysectomy	Male	35	75	0.10†	±0.043
Hypophysectomy	Female	4	11	0.08	
Hypophysectomy + DOCA	Male	27	55	0.10	±0.034
Hypophysectomy + Cortisone	Male	20	58	0.13	±0.037
Hypophysectomy + ACTH	Male	12	26	0.11	±0.036
Hypophysectomy + growth hormone, testosterone, and thyroxin	Male	9	20	0.15‡	±0.045

* Protein excretion is expressed as mgm./hr./100 cm.² body surface in order to allow direct comparison of protein excretion in small animals with the protein excretion of larger rats having larger kidneys.

† Significantly lower than level of proteinuria in normal male rats ($p = <0.01$).

‡ Only result significantly elevated above the level of proteinuria in hypophysectomized males ($p = <0.01$).

proteinuria as the "base proteinuria" in contrast to the massive "renin proteinuria." The base protein excretion of the hypophysectomized male rat falls to the normal female level. In the few females studied, no apparent change in the originally low level of base proteinuria occurred following hypophysectomy.

From Table 2, it may be seen that neither DOCA, cortisone, nor ACTH

significantly elevated the depressed level of base proteinuria in the hypophysectomized rat. A significant restoration of proteinuria did occur however, when a combination of growth hormone, thyroxin, and testosterone propionate were administered.

III. *Effect of hypophysectomy on organ weights*

In Table 3 are summarized the mean organ weights found at autopsy in each of the groups tested above. In addition to the actual organ weight,

TABLE 3. ORGAN WEIGHTS OF HYPOPHYSECTOMIZED RATS SACRIFICED 24 DAYS POSTOPERATIVELY

Hypophysectomized rats Group	No. of rats	Body wt. gms.	Rate body wt. loss after hyp. gms./day	Kidneys gms.	Adrenals mg.	Gonads gms.	Heart gms.	Spleen gms.
I. No treatment	11	180	−1.9 ±0.79*	0.98 ± 0.086	13.5 ± 2.64	0.61 + 0.194	0.55 ± 0.050	0.67 ±0.166
% deviation from predicted normal				*−22.1*	*−49.4*	*−72.2*	*−12.4*	
II. DOCA	15	187	−2.0 ±0.41	1.02 ± 0.210	11.5 ± 2.60	0.58 ± 0.148	0.55 ± 0.063	0.66 ±0.151
% deviation from predicted normal				*−14.5*	*−57.7*	*−73.8*	*−14.8*	
III. Cortisone	14	163	−3.5 ±0.64	1.12 ± 0.166	11.8 ± 2.48	0.45 ± 0.301	0.61 ± 0.094	0.23 ±0.090
% deviation from predicted normal				*− 3.8*	*−53.7*	*−77.3*	*+ 4.0*	
IV. ACTH	4	183			13			
% deviation from predicted normal					−52.0			
V. Growth hormone, thyroxin, and testosterone**	7	211	+0.4	1.21 ± 0.063	12.7 ± 3.15	1.73 ± 0.298	0.65 ± 0.080	0.96 ±0.217
% deviation from predicted normal				*−14.7*	*−56.1*	*−25.2*	*− 7.9*	

* Figures preceded by ± represent standard deviation.
** This group was sacrificed seventeen days postoperatively.

the per cent change from the expected organ weight found in normal rats of the same body weights (Addis and Gray, 1950) is expressed. Total body weights showed a significantly greater decline following hypophysectomy in cortisone-treated rats than DOCA-treated or untreated animals. The group receiving growth hormone, thyroxin, and testosterone propionate, however, gained an average of 7 gm. per rat by the seventeenth day following hypophysectomy. Adrenal glands were atrophied in all groups. The size of the testes was markedly diminished in all animals except those receiving growth hormone, thyroxin, and testosterone propionate. Spleens[7] were very small in rats receiving cortisone, but less small in untreated rats and rats

[7] Caution must be exercised in interpreting spleen weight changes in our colony since it is known to have latent Bartonella muris infection.

receiving DOCA. Spleens were significantly larger in rats receiving growth hormone, thyroxin, and testosterone propionate. Heart weights were subnormal in untreated and DOCA-treated rats. Despite the greater body weight loss found in cortisone-injected rats, heart weights fell within the normal expected range. Kidney weights were greatly diminished in untreated hypophysectomized rats. In DOCA-treated and cortisone-treated animals, absolute kidney weights were not significantly higher. Again, when related to the body weight, kidney weights fell within the normal expected range in the cortisone-treated rats but not the DOCA-treated animals. The growth hormone, thyroxin, testosterone group had significantly larger kidneys then the other groups, although they were still below the normal expected kidney weights for their increased body weights.

DISCUSSION

One might question whether it is actually the renin in our preparation of kidney extract which is responsible for the production of proteinuria. In our experience, four pressor units produced the same degree of proteinuria irrespective of the purity of the renin preparation. Thus, administration of a crude extract of renin, in which the four pressor units were contained in 4 mg. of protein, produces the same degree of proteinuria as the most pure extract tested in which the four pressor units were contained in 0.025 mg. of protein.

The results presented demonstrate that hypophysectomy, like adrenalectomy, abolishes the proteinuria response to renin in the rat. If the abolition of proteinuria by hypophysectomy is due to diminished adrenal cortex activity, then adrenal steroid administration should restore renin proteinuria in the hypophysectomized rat just as it does in the adrenalectomized animal. Cortisone, in fact, largely restores this proteinuria in the hypophysectomized rat. DOCA however, does not. Why these two steroids, equally effective in restoring proteinuria in the adrenalectomized rats, exhibit such a marked difference in effectiveness in hypophysectomized rats is not at present clear.

ACTH fails to restore the proteinuria response to renin in the hypophysectomized rats. This might be attributed to a lack of response of our rats to ACTH in the dosage used. In support of this view, we have found that rats of our colony require three successive doses of 9.6 mg. of ACTH (Armour Standard equivalent) to produce a satisfactory eosinopenia. Further, the dosage administered in the present experiment (2.6 mg. of Armour Standard equivalent given twice daily) was unable to prevent the adrenal cortex atrophy which occurs following hypophysectomy (Table 3). Against this hypothesis, however, we have not been able to effect any significant restoration of renin proteinuria even in acute experiments. When

animals received 30 mg. of ACTH daily for 48 hours and 72 hours after hypophysectomy, at which time they were injected with four units of renin, they still showed no consistent restoration of proteinuria.

The untreated hypophysectomized rat loses weight steadily on the stock diet. We do not believe that changes secondary to their poor nutrition abolish the proteinuria response to renin, for cortisone administration restores renin proteinuria while exaggerating the progressive weight loss. Although the administration of growth hormone, thyroxin, and testosterone propionate was successful in enabling the hypophysectomized rats to show a slight weight gain in the seventeen days between hypophysectomy and termination of the experiments, these animals were incapable of responding to renin injection with increased proteinuria.

As far as its effect on spontaneous male proteinuria is concerned, hypophysectomy is again similar to adrenalectomy in that it diminishes the base proteinuria. In contrast to adrenalectomy however, neither DOCA nor cortisone administration resulted in a rise in the depressed level of base proteinuria. Administration of growth hormone, thyroxin, and testosterone propionate did increase the depressed base proteinuria significantly. Growth hormone has been shown (White, Heinbecker, and Rolf, 1949) to raise depressed renal clearances in hypophysectomized dogs, and it is possible that an increased filtration rate of protein accounts for the increased base proteinuria found in animals receiving growth hormone, thyroxin, and testosterone propionate.

Lewis, Rosemberg, and Wilkins (1950) have observed an increased rate of weight loss in hypophysectomized rats receiving cortisone. Our results confirm this effect of cortisone and further indicate that no such increase in rate of weight loss occurs in hypophysectomized rats receiving DOCA.

The absolute kidney weights do not differ significantly in cortisone-treated and DOCA-treated rats from those of untreated hypophysectomized animals. The finding that the cortisone-treated rats have larger kidneys in relation to body weight may reflect either a diminished rate of kidney weight loss or an increased loss of weight of other body constituents (such as fat). In the absence of detailed studies of the carcass constituents no conclusions can be reached concerning this point.

SUMMARY

1. Hypophysectomy abolishes the proteinuria produced by the intraperitoneal injection of renin in the rat. Cortisone administration restores the proteinuria response to renin. DOCA and ACTH, in the dosage used, did not restore renin proteinuria.

2. Spontaneous male ("base") proteinuria is diminished significantly by hypophysectomy. Neither DOCA, cortisone, nor ACTH administra-

tion appreciably restores this proteinuria. Administration of a combination of growth hormone, thyroxin, and testosterone propionate significantly restored the diminished base proteinuria.

3. Cortisone administration to hypophysectomized rats increases the rate of body weight loss. DOCA produces no increase in the rate of body weight loss over untreated hypophysectomized rats.

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THE RELATION OF THE ADRENAL TO BLOOD FORMATION IN THE RAT[1]

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THE possibility of an endocrine relation to certain processes of hemopoiesis has been recognized for some time. The earlier clinical papers reporting peripheral hematologic alterations as an accompaniment of pituitary (Silver, 1933), thyroid (Emery, 1923), or adrenal (Rowe, 1929) hypofunctional states have been given additional credence in experiments performed in the laboratory animal (see reviews by Gordon and Charipper, 1947, and Daughaday, Williams and Daland, 1948). The evidence is also clear that adequate replacement therapy will prevent or overcome many of the hemic changes characteristic of endocrine dysfunction (Meyer, Thewlis and Rusch, 1940; Vollmer and Gordon, 1941; Vollmer, Gordon and Charipper, 1942; Crafts, 1946a, b).

Impetus to further research in this field has been provided of late by the findings that adrenal cortical hormones exert an influence upon the numbers and types of white cells in the peripheral blood (Dougherty and White, 1944; White and Dougherty, 1945; Forsham, Thorn, Prunty and Hills, 1948) and by the recent application of the adrenal cortical and adrenocorticotrophic factors to the treatment of leukemic conditions in man (Pearson and Eliel, 1950).

Despite the steadily increasing interest being paid to the influence of adrenal cortical principles upon the blood elements, surprisingly little effort has been made to determine the mechanisms underlying these effects. It was thought that a step in the solution of this problem might be provided through a detailed correlation of the peripheral blood changes to the bone marrow cytology in the adrenal insufficient animal, with and without replacement therapy. The results of one phase of this study will be described in the present paper. Preliminary reports of some aspects of this work have already been published (Gordon and Piliero, 1950; Piliero, Landau and Gordon, 1950).

Received for publication May 28, 1951.

[1] Supported by a grant from the Dazian Foundation for Medical Research.

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MATERIALS AND METHODS

Adrenalectomized young adult female rats of a hardy, closely inbred strain, approximately 3 months of age and weighing 140–170 gm. were employed. Sham-adrenalectomized rats constituted a part of the control groups. All animals were fed a standard laboratory ration and kept at temperatures of 70–80 degrees F. The adrenalectomized rats were maintained on 1% NaCl given as drinking water.

Peripheral blood from the tail was employed for all procedures. Red and white cell counts were made in duplicate; the former were required to agree within ±4% and the latter to within ±10%. Differential counts were made from smears stained with Wright's. In some of the groups, eosinophiles were estimated by the method of Randolph (1949). Reticulocyte counts were made from wet mounts of whole blood suspended in 2% sodium citrate solution and stained with brilliant cresyl blue. One thousand cells were counted and the reticulocytes expressed as a percentage of these. Hemoglobin concentrations were determined by the acid hematin method with a photoelectric colorimeter. The method of Straus and Faigen (1946) was used to ascertain red cell fragilities in hypotonic saline. Fragility values are given in terms of percentage of cells lysed in 0.45% saline. Sedimentation rates, expressed as mm. fall per hr., were determined by the micromethod of Landau (1933). Duplicate hematocrit determinations from heparinized blood,

TABLE 1. PLAN OF EXPERIMENTAL TREATMENTS IN ADRENALECTOMIZED RATS

Group	1	2	3	3a	3b	4	5	6	7	8	9	10	11
No. of rats	12	12	12	5	4	12	5	5	3	4	5	6	6
Hormonal treatment	Sham adrenx.	None	A.C.E.[1]	A.C.E.[2] conc.	A.C.E.[3]	Epin.[4] (1–10,000)	Epin. (1–4 mill.)	DOCA[5]	Comp. A[6]	Comp. E[3]	Comp. E	Comp. E	Demedull.
Daily dosage	6 rats rec'd. 1–2 cc. Saline	—	1–2 cc.	2 cc.	0.5 cc.	2 cc. in saline	2 cc. in saline	0.5 mg.	0.5 mg. in saline suspension	0.5 mg.	1.0 mg.	3.0 mg.	—

[1] Adrenal cort. ext. (Comp. E equiv.—164 μg./cc.) supplied by Dr. D. A. McGinty, Parke, Davis and Co.
[2] Adrenal cort. ext. concentrate (Comp. E equiv.—480 μg./cc.) supplied by Dr. D. A. McGinty, Parke, Davis and Co.
[3] Lipo-adrenal cortex (Upjohn and Co.).
[4] Adrenalin hydrochloride (Parke, Davis and Co.)
[5] Desoxycorticosterone acetate, furnished by Dr. F. Yonkman, Ciba Pharm. Prods., Inc.
[6] 11- and 17-OH-11-dehydrocorticosterone acetate supplied by Drs. H. J. Robinson and A. Gibson, Merck and Co., Inc.

spun at 7,000 RPM for 15 minutes, were made by a capillary micromethod and were required to agree to within ±1 mm. Control hematologic determinations were carried out in all animals before experimental treatment was begun. The experimental groups were established as follows:

Series I. *Adrenalectomized and control animals.* Hematologic studies were conducted in 12 sham-adrenalectomized control (Group 1) and in 12 adrenalectomized (Group 2) animals at weekly intervals for a period of 4 weeks after operation. An additional 24 adrenalectomized rats served as controls to the various groups undergoing hormonal treatment in Series II.

Series II. *Replacement therapy.* All animals in this series were completely adrenalectomized except for a group which was subjected to adrenal demedullation only. Hormonal treatments, indicated in Table 1, were administered as daily subcutaneous injections for a period of 14 days beginning with the day of operation. The animals in Groups 3 and 4 were continued on daily treatment for an additional 2 weeks. Hematologic examinations were made at weekly intervals.

At the termination of the observation periods, all rats were anesthetized lightly with ether and exsanguinated by cardiac puncture. The blood was allowed to clot at room temperature, stored in a refrigerator for 12 hours and the serum collected. Both femora

and tibiae were dissected, cracked open and the bone marrow removed and placed in a watch glass containing homologous serum obtained from previously killed rats. The marrow was prepared in suspension form by drawing it up and down gently in the serum with a glass pipette. Smears were then made for determination of the myelograms. The smears were fixed immediately in absolute methanol for 5 minutes and stained with Wright's or Giemsa. When the latter was used, a 1:50 dilution buffered at pH 5.8 (Endicott, 1945) gave the best differentiation. For each animal, 1500 nucleated cells were counted and classified according to the criteria suggested by Endicott and Ott (1945). Some bone marrow material from the proximal end of the femur of each animal was fixed in Downey's fluid, sectioned serially at 5μ and stained with either hematoxylin and eosin or by Wright's technique.

Weekly body weight measurements were made for all animals of both Series I and II.

The technique of Speirs and Meyer (1949) was adopted to test the completeness of the operation or the presence of adrenal cortical accessory tissue in the particular strain of rats employed. A group of 10 rats was adrenalectomized, maintained on 1% saline and, at 4 weeks following the operation, injected with epinephrine (20 μg./100 gm. body

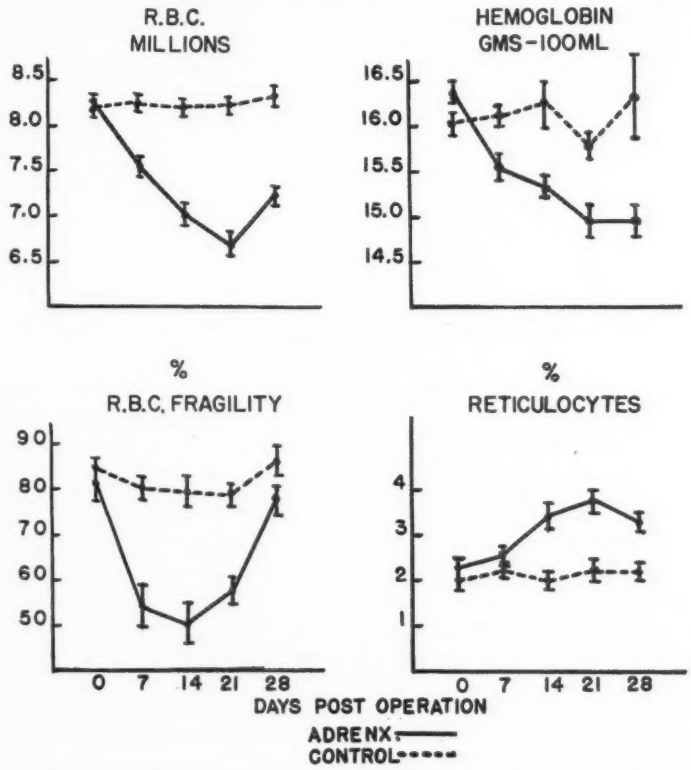


FIG. 1. Erythrocytic alterations induced by adrenalectomy (12 rats) and by sham-adrenalectomy (12 rats). Vertical lines through mean values represent ± 1 standard error of the mean.

wt.). Eosinophile counts (chamber method) were made just prior to and 4½ hours after administration of the drug. Saline treatment was then withdrawn. Approximately one month later, to test resistance to stress, the 5 surviving rats as well as 5 sham-operated controls were injected subcutaneously with 2 cc. of 10% formalin. Times of death following the injections were then recorded.

RESULTS

Series I

A. Peripheral blood

The effects of adrenalectomy upon the peripheral blood picture of the female rat are shown in Fig. 1. It will be noted that this operation results in reductions in red cell count, hemoglobin concentration and red cell fragility which become maximal at approximately 14–21 days following adrenal ablation. These values then begin to rise but completely normal levels are not attained up to 60 days following the operation. Reticulocyte values display an increase, reaching maximal levels at 21 days subsequent to adrenal removal. Thereafter they tend to fall and become normal by

TABLE 2. INFLUENCE OF ADRENALECTOMY UPON SOME ERYTHROCYTIC VALUES (MEANS ± STANDARD ERRORS IN 12 RATS)

Days after operation	Color index	Volume index	Hematocrit (%)	Mean Corp. Vol. (cu.μ.)	Mean Corp. Hb (μ.μg.)	Mean Corp. Hb Conc. (%)
0	1.01 ± 0.01	1.00 ± 0.01	43.6 ± 0.5	52.6 ± 0.7	19.8 ± 0.1	38.1 ± 0.5
7	1.04 ± 0.01	1.03 ± 0.01	41.0 ± 0.6	54.2 ± 0.8	20.6 ± 0.3	38.2 ± 0.7
14	1.10 ± 0.02	1.05 ± 0.02	38.1 ± 1.0	54.3 ± 1.2	21.9 ± 0.2	40.5 ± 0.9
21	1.13 ± 0.02	1.12 ± 0.02	39.6 ± 0.4	58.9 ± 1.1	22.3 ± 0.3	37.9 ± 0.2
28	1.05 ± 0.02	1.08 ± 0.02	40.7 ± 0.4	56.1 ± 0.9	20.7 ± 0.4	36.8 ± 0.7

approximately 60 days. Sedimentation rates increase slightly and remain above normal for 8 weeks after adrenalectomy. No significant alterations in the peripheral total and differential white cell counts are induced by adrenal removal at 1 week and up to 60 days postoperative.

Table 2 indicates that the color index, volume index, mean corpuscular volume and mean corpuscular hemoglobin display significant rises after adrenalectomy, attaining highest values at 3 weeks, following which they decrease gradually, approaching normal levels at 60 days. Mean corpuscular hemoglobin concentration values are unaltered by adrenalectomy. No changes in these values are observed in the sham-operated and unoperated controls.

B. Bone marrow studies

Observation of Table 3 reveals that significant alterations in the bone marrow myelograms are produced by adrenal removal. By as early as the first week, there occurs an increase in the percentages of total granulocytes

accompanied by a decrease in the percentages of nucleated erythroid elements, thus resulting in a fall in the erythroid-myeloid cell ratio. These alterations become maximal at 2 weeks following adrenal removal. The rise in granulocytic population observed at this time is due to increases in the

TABLE 3. EFFECTS OF ADRENALECTOMY AND REPLACEMENT THERAPY ON BONE MARROW CELLULAR PERCENTAGES[1] (MEANS ± STANDARD ERRORS)

	No. of rats	Blasts	Neutrophiles[2]		Eosinophiles	Total Granulocytes	Nucleated Erythrocytes	Erythroid-Myeloid Ratio
			Young	Mature				
Controls	14[3]	1.6±0.1	10.9±0.6	21.1±1.4	9.1±0.8	41.6±2.4	56.5±2.1	1.46±0.13
Adrenx. 1 week	4	1.4±0.4	12.2±1.4	29.2±1.5	10.7±1.7	52.0±3.7	46.1±3.0	0.91±0.12
2 week (Group 2)	8	3.2±0.5	17.1±1.1	24.6±0.7	14.2±0.8	55.9±1.8	40.5±2.2	0.73±0.07
3 week	6	1.6±0.7	18.8±1.3	28.3±2.8	9.1±1.8	51.9±3.0	46.3±3.3	0.95±0.18
4 week	5	1.0±0.4	19.3±0.2	20.0±2.1	9.8±1.2	49.1±2.0	49.8±2.3	1.01±0.10
A.C.E.[4] (Group 3)	12	0.5±0.1	18.5±1.4	24.9±2.0	12.2±1.2	55.6±2.7	44.1±2.8	0.84±0.10
A.C.E. (Group 3a)	5	0.3±0.1	10.5±0.8	20.8±1.3	11.2±2.7	42.5±3.1	56.2±3.2	1.36±0.16
A.C.E. (Group 3b)	4	0.6±0.2	10.9±2.4	21.8±3.5	11.7±2.3	43.7±4.9	54.7±4.7	1.36±0.25
Epineph. (Group 4)	8	0.6±0.2	11.7±1.7	32.9±1.6	12.3±0.8	56.8±2.6	42.1±2.6	0.77±0.08
DOCA (Group 6)	5	0.3±0.1	4.2±0.5	30.5±1.1	16.0±2.0	50.6±2.6	48.8±2.4	0.98±0.12
Comp. E (Group 8)	4	0.8±0.2	6.6±1.0	36.1±1.8	9.0±1.8	51.6±4.3	47.5±4.2	0.96±0.17
Comp. E (Group 9)	5	0.8±0.1	6.3±1.3	39.4±3.5	8.2±1.9	53.7±5.1	43.7±4.2	0.85±0.16
Comp. E (Group 10)	6	0.7±0.1	12.6±0.7	27.5±1.9	3.4±0.5	43.5±1.7	54.1±2.5	1.29±0.17
Demedull. (Group 11)	6	0.9±0.2	10.2±2.2	21.8±0.9	11.9±1.2	45.1±3.8	52.8±4.3	1.25±0.25

[1] Lymphoid elements are not included in the myelograms; they were unaffected by the various experimental treatments employed and did not exceed 5% of the total population of marrow cells in any of the groups.
[2] Young neutrophiles included promyelocytes and myelocytes; mature neutrophiles include metamyelocytes and segmented forms.
[3] Includes 6 unoperated and 8 sham-operated rats (grouped together because of absence of significant difference between the 2).
[4] Treatment in Groups 3–10 (see Table 1) was begun on the day of ardenalectomy and continued daily for 2 weeks at which time the myelograms were determined. Values for these groups should be compared to those for Group 2 (2 week adrenx. rats).

concentration of all myeloid cell components, including eosinophilic and young and mature neutrophilic forms. Although a tendency is seen for the total granulocytic cell percentages to fall and the nucleated erythrocytes to rise at the third week, normal values of the myelogram are not attained at this time or at 4 weeks following adrenalectomy. Thus the percentages of promyelocytes and myelocytes one month after adrenal removal are as high as those noted at the 2 week period. By the fourth week, however, the levels of eosinophilic and mature neutrophilic components have returned to normal.

The observations on the bone marrow sections parallel those made on

the smears. Thus an increase in the myeloid and a decrease in erythroid cell relative numbers are observed at the 1st and 2nd weeks after adrenalectomy (Figs. 3 and 4). Some vacuolization of the marrow, greater than that seen in the controls, is evident in the 2 week animals. At the 3rd and 4th weeks after adrenal removal, myeloid cell elements still predominate in the marrow sections, although their concentration appears less than that noted in the 2 week-adrenalectomized rats.

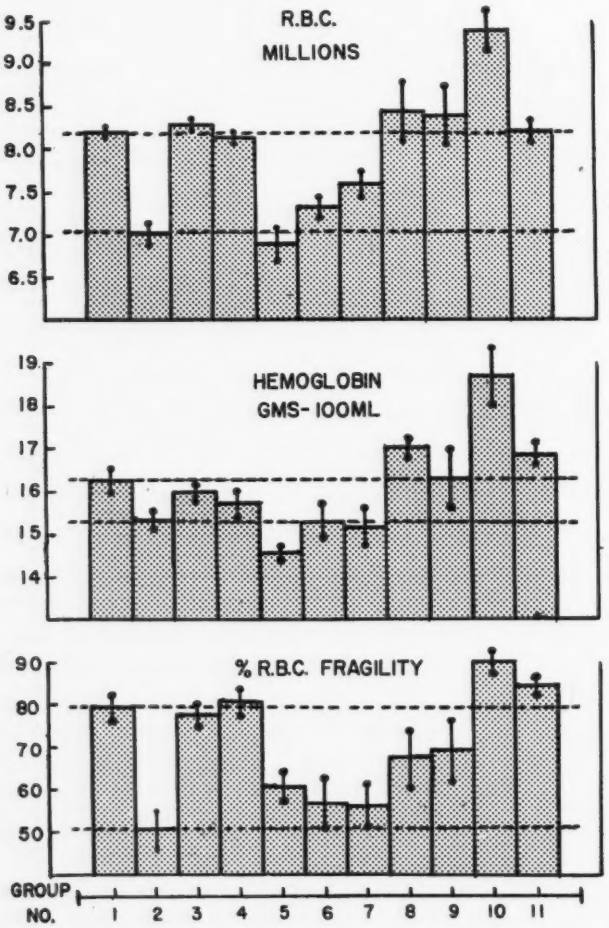


FIG. 2. Erythrocytic alterations in rats undergoing experimental treatment for 2 weeks following operation. Vertical lines represent ±1 standard error of the mean. Top horizontal broken line indicates value in control and bottom horizontal broken line indicates value in adrenalectomized rats. See Table 1 for description of groups.

Series II

A. Peripheral blood

Figure 2 depicts the effects of various hormonal treatments upon some peripheral red blood cell values in adrenalectomized rats. It will be noted that the alterations in red cell count, fragility and hemoglobin concentration induced by adrenal removal are prevented by daily administration of adrenal cortical extract or by epinephrine (200 μg.). Since epinephrine in a concentration of 1–4 million appears as a contaminant in the aqueous adrenal cortical extract used (personal communication from Dr. D. A. McGinty), this dose was used as a control (Group 5). No significant changes in red cell numbers, fragility or hemoglobin values are obtained with this amount (0.5 μg.) of the medullary hormone. Thus the effects of the adrenal cortical extract used (Group 3) cannot be explained by its epinephrine content. DOCA and Compound A, in dosages of 0.5 mg., are largely ineffective in overcoming the erythrocytic alterations in the adrenalectomized animal. Compound E, in dosages of 0.5 and 1.0 mg., successfully prevents the drop in red cell count and hemoglobin but is ineffective in restoring red cell fragilities to normal levels. Significantly greater than normal fragility values as well as higher red cell counts and hemoglobin concentrations are obtained with the 3 mg. dosages of Compound E. Adrenal demedullation is without effect upon the various values studied.

The increases in color index, volume index, mean corpuscular volume and hemoglobin, observed at 14 and 21 days following adrenalectomy are prevented by adrenal cortical extract, epinephrine (200 μg.) and by Compound E, in dosages of 1.0 and 3.0 mg. daily. DOCA, Compounds A and E, given in amounts of 0.5 mg., do not effectively prevent these alterations. Epinephrine in a concentration of 1–4 million and demedullation are also ineffective treatments.

Save for 2 exceptions, no significant alterations in the peripheral total and differential white cell counts are produced by adrenal cortical extract, epinephrine, DOCA, Compound A and by Compound E, in daily amounts of 0.5 and 1.0 mg., when counts are made at weekly intervals after adrenalectomy. The exceptions include a tendency for neutrophilia and lymphopenia to be present after 7 days in the rats receiving 200 μg. epinephrine (Group 4) and in those injected with aqueous adrenal cortical extract concentrate (Group 3a). These effects are no longer apparent after 2 weeks of treatment. A slight degree of eosinopenia is also seen in the peripheral blood of the rats receiving Compound E in the 0.5 and 1.0 mg. daily doses. As observed in Table 4, Compound E, in the 3 mg. dosages, produces, at both 7 and 14 days, significant reductions in the lymphocytic and eosinophilic counts and increases in the neutrophilic cell levels.

TABLE 4. EFFECTS OF COMPOUND E UPON DIFFERENTIAL WHITE CELL COUNTS OF ADRENALECTOMIZED RATS (MEANS ± STANDARD ERRORS)

Daily dosage (mg.)	Days of treatment	Total WBC thous. cu. mm.	Lymphocytes %	Neutrophiles %	Eosinophiles[1] cu. mm.
0.5	0	13.4 ± 0.2	83.5 ± 1.2	13.8 ± 0.9	320 ± 71
(4)[2]	7	11.5 ± 0.5	81.0 ± 2.4	16.5 ± 2.2	264 ± 48
	14	11.8 ± 0.3	84.6 ± 2.6	13.5 ± 2.1	206 ± 39
1.0	0	11.7 ± 1.0	81.2 ± 2.7	16.7 ± 2.2	234 ± 41
(5)	7	13.9 ± 0.8	80.8 ± 2.3	17.8 ± 2.2	119 ± 40
	14	12.4 ± 0.7	78.8 ± 3.4	19.6 ± 3.3	127 ± 36
3.0	0	12.0 ± 0.6	80.8 ± 4.4	16.0 ± 3.1	335 ± 60
(6)	7	15.2 ± 2.2	62.3 ± 4.5	36.3 ± 4.6	120 ± 31
	14	10.3 ± 0.7	62.7 ± 2.6	36.8 ± 2.7	116 ± 38
Saline	0	10.6 ± 0.3	81.0 ± 3.1	15.6 ± 3.0	316 ± 55
(8)	7	11.9 ± 0.8	80.0 ± 2.5	17.8 ± 2.2	209 ± 41
	14	12.3 ± 0.6	76.8 ± 2.8	20.3 ± 2.3	342 ± 87

[1] Chamber method.
[2] Number of rats.

B. Bone marrow

Table 3 reveals that in the dosages employed, aqueous adrenal cortical extract (Group 3) exerts no influence upon the bone marrow myelogram of the adrenalectomized rat. However, treatment with both the cortical extract concentrate (Group 3a) and lipo-adrenal extract (Group 3b) does result in a significant decrease in the percentages of granulocytes (especially younger forms) and elevates the percentages of nucleated erythroid cells. Erythroid-myeloid cell ratios are increased almost to the levels seen in the sham-operated controls. Epinephrine (200 μg.), DOCA (0.5 mg.) and Compound E (0.5 and 1.0 mg.) significantly decrease the percentages of immature neutrophilic forms and increase the numbers of metamyelocytes and segmented forms. Total granulocytic percentages, however, remain unaffected. Although these 3 agents, in the dosages mentioned above, tend to increase slightly the nucleated erythrocytic percentages, the erythroid-myeloid cell ratios remain below 1.0. Of the three, only Compound E effectively prevents the eosinophilia characteristic of the marrow of the adrenalectomized rat. When Compound E is administered in daily amounts of 3 mg., the marrow myelogram is restored to approximately normal values, save for an accompanying marked eosinopenia. Demedullation does not appear to result in any significant departure of the myelogram from normal values.

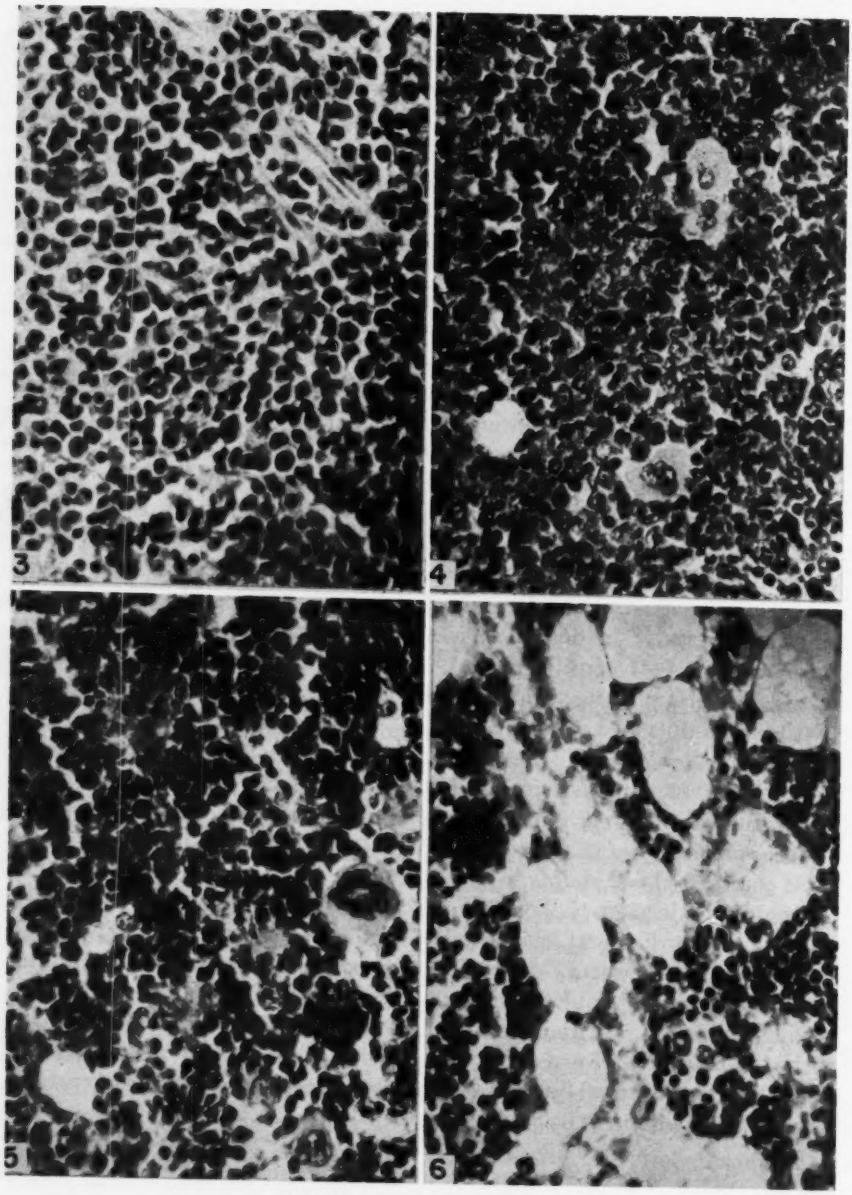
On the whole, the results of the marrow section studies correlate well with the myelograms obtained from smears, Thus, definite increases in the proportions of erythrocytic precursors are observed in the marrow sections of the adrenalectomized rats which received the cortical extract concentrates (Fig. 5). With the 0.5 and 1.0 mg. dosages of Compound E, the results are quite variable. Increased percentages of erythroid cells appear in some sections but not in others. Some increased vacuolization is observed in the marrow sections of the rats receiving 1.0 mg. dosages of Compound E. The sections of the adrenalectomized rats receiving 3.0 mg. of the hormone daily display a considerable reduction in total cellularity due to increased vacuolization (Fig. 6). Nevertheless, the remaining marrow cells are predominantly of the erythroid type.

Body weights. At 2 weeks following the operation, body weight gains experienced by the salt-maintained adrenalectomized rats are somewhat smaller (+10 gm. in 19 animals) than those seen in the sham-operated controls (+15 gm. in 12 animals). By the 3rd week, however, the weight gains are similar in the two groups (+20 gm. in both groups). Epinephrine (200 μg.) and DOCA inhibit slightly the body weight gains displayed by the salt-maintained adrenalectomized rats at 2 weeks subsequent to operation. Compound E, in dosages of 0.5–1.0 mg., prevents the body weight gains and, in the 3 mg. dosage, causes significant weight loss in the adrenalectomized rat (−15 gm. in 6 rats). The growth rates of the adrenalectomized rats receiving the various cortical extracts are similar to those shown by the sham-operated controls.

Tests for completeness of operation. At 4 weeks following adrenal removal, none of the 10 animals employed to test for the presence of additional tissue, displayed an eosinopenic response following epinephrine injections. On the contrary, 9 of the 10 rats responded with a significant rise in eosinophile count. Red cell counts in these 10 rats, as determined just before the epinephrine injections, had returned to approximately normal levels. Five of these 10 rats died within a period of a month after withdrawal of the 1% saline as drinking water. Macroscopic signs of regenerating adrenal cortical tissue were not visible in these rats at autopsy. Four of the 5 surviving adrenalectomized rats and none of the sham-operated controls succumbed within 24 hours after formalin injections. One of the 4 which died displayed a small adrenal cortical body near the right kidney. No evidence of cortical tissue was apparent in the lone survivor.

DISCUSSION

The finding of a mild peripheral anemia which attains its peak at approximately 2 weeks following adrenal removal is in accord with the results obtained previously by Crafts (1941). Although the salt-maintained adrenal-



FIGS. 3–6. See next page for description.

ectomized rats eat and gain weight for the first 2 weeks after operation at a somewhat subnormal rate, it is doubtful that this slight degree of malnutrition can account for the peripheral anemia since both paired-fed and paired-weight controls show no such reductions in red cell count (unpublished results). It would seem likely that the anemia is the result of the reduced erythroid cell concentrations noted in the bone marrow of these animals. Although the anemia in the Addisonian patient appears to be normochromic and normocytic (Báez-Villaseñor, Rath and Finch, 1948), the present results indicate that in the adrenalectomized rat it is of the hyperchromic macrocytic variety. The increase in red cell resistance which accompanies the anemia has been demonstrated to be due to an augmentation in the concentration of certain, as yet unidentified, plasma factors inhibitory to hypotonic saline lysis (Megel and Gordon, 1951). Correlated possibly to the observation that a decrease in red cell fragility occurs after adrenalectomy is the recent finding by Hamilton *et al.* (1951), that this operation results in a reduction in plasma iron, correctable by administration of adrenal cortical factors. The significance of the reticulocytosis following adrenalectomy is not clear. That it is probably not associated with increased red cell destruction is seen from unpublished findings that the serum bilirubin levels in the rat are not increased by adrenal removal.

In addition to the observed lowered concentration of erythrogenic elements, the marrow of the adrenalectomized rat is characterized by an increase in the percentages of myeloid cells. All white cell elements appear to participate with the most striking increases noted in the numbers of immature neutrophilic and total eosinophilic cells. This tendency for a leukemoid state to develop in the marrow of the adrenalectomized rat is not paralleled by significant alterations in the numbers and types of white cells in the peripheral blood. Crafts (1941) also has observed no peripheral white cell changes in the salt-treated adrenalectomized rat.

Although all forms of replacement therapy used, except DOCA and

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Figures 3–6 represent sections of rat bone marrow fixed in Downey's fluid and stained with hematoxylin and eosin. 430×.

FIG. 3. Sham-adrenalectomized control. Observe areas of erythroid cells (darkly stained) interspersed among myeloid elements.

FIG. 4. Two weeks after adrenalectomy. Myeloid cell concentration is greater than in control.

FIG. 5. Adrenalectomized rat treated daily for 2 weeks with 0.5 cc. lipo-adrenal cortex. Areas of proliferating erythrogenic cells are conspicuous (cf. Fig. 4).

FIG. 6. Adrenalectomized rat treated daily for 2 weeks with 3.0 mg. Compound E. Considerable vacuolization is apparent. Remaining marrow is essentially normal in relative erythroid-myeloid cell concentrations.

Compound A, succeed in preventing the peripheral erythrocytic alterations in the adrenalectomized rat, only the cortical extract concentrate, lipoadrenal cortex and Compound E (3.0 mg.) restore the bone marrow myelograms to nearly normal levels. On the other hand, DOCA, which in 0.5 mg. dosages is without effect on the peripheral picture, does induce some change in the marrow myelogram, evoking an increase in the concentration of mature myeloid components. This lack of correlation, noted in several of the replacement therapy groups, between the peripheral and marrow cell pictures, should not be altogether surprising, for it is apparent that the concentrations of blood cells in the peripheral circulation are dependent not only upon the rate and type of cell production by the marrow, but also on total functional marrow mass, rate of release of cells from this and other organs, longevity of the cells in the circulation, rate of removal of cells from the circulation, their destruction, or possible redistribution to other organs (Opie, 1904; Steinberg and Martin, 1950). Any or all of these could be affected conceivably by the various endocrine procedures employed in the present experiments.

It is of interest that epinephrine, in daily dosages of 200 μg. prevents the peripheral erythrocytic changes in the adrenalectomized animal. Evidence that the medullary hormone produces effects on both red and white blood cell numbers independently of the adrenal and/or spleen has appeared in the literature on several occasions (Lucia, Leonard and Falconer, 1937; Davis, 1942; Gabrilove, Volterra, Jacobs and Soffer, 1949; Dury, 1950; Strand and Gordon, 1950; Stein, Martin and Falk, 1951). The more striking effects induced by epinephrine upon the peripheral white cells of the adrenalectomized rat in the acute experiment (Dury, 1950; Stein, Martin and Falk, 1951) are not seen, for the most part, in the chronic studies described here. Nevertheless, alterations are observed in the ratio of young to mature neutrophilic cells in the marrow myelograms of the adrenalectomized rats treated with this factor. A normal physiologic role for epinephrine in blood formation processes, however, is as yet to be established, especially in view of the observation that demedullation of the adrenal is without significant influence upon any of the values examined in the present study.

Not reported previously is the present observation that eosinophilia occurs within the bone marrow of the salt-maintained adrenalectomized rat and that this may be prevented by Compound E in daily doses of 0.5–1.0 mg. With larger doses (i.e., 3.0 mg.) the marrow may actually be rendered eosinopenic. This action upon the marrow may account, to an extent, for the peripheral eosinopenia produced by Compound E, as reported here and by numerous others. It has been stated (Dougherty, 1950) that the cortical steroids induce peripheral eosinopenia by causing these

cells to emigrate from the circulation into the tissues. This possibility, as well as others, including uneven distribution in the vascular bed, increased destruction of the cells, or elimination through waste channels requires further investigation.

The increased vacuolization observed in the marrow sections of the adrenalectomized rats treated with the higher doses of Compound E is probably associated with excessive fat infiltration into this organ. A similar observation has been reported by Baker and Ingle (1948) in the tibial marrow of rats treated with ACTH. The tendency for the quantity of body fat to be increased, associated with inhibition of body weight gains or actual loss of body weight, is becoming recognized as a constant feature of overdosage with Compound E (Winter, Silber and Stoerk, 1950). Yet despite the vacuolization observed in the sections, the marrow smears indicate that the remaining cellular types, in the Compound E-treated adrenalectomized rats, are maintained at approximately the levels characteristic of the sham-operated controls. This reduced volume of functional marrow is apparently capable of furnishing quantities of erythrocytes to the peripheral circulation sufficient to maintain normal red cell counts. It is unlikely that hemoconcentration is a significant factor here in view of the report by Winter, Silber and Stoerk (1950) that serum protein concentrations show no change in the rat treated chronically with high dosages of Compound E. The constancy of the total peripheral white cell counts in the these rats would also argue against this interpretation.

Of especial significance in this report is the tendency for the majority of the formed blood cell values in the adrenalectomized animal to display their most significant deviations from normal at approximately 2–3 weeks following the operation and then to return gradually towards the control levels. The possibility of the presence of adrenal cortical accessories or regeneration of adrenal cortical tissue has been considered but little evidence for this was noted at autopsy. Moreover, the tendency for the 4 week-adrenalectomized rats to display a peripheral eosinophilia after epinephrine administration, the death of half of the rats after salt withdrawal and the succumbing of the majority of the remaining animals to an intensity of formalin stress insufficient to kill the controls would also indicate the absence of significant quantities of cortical tissue in these animals. It would appear thus that certain, as yet incompletely understood, adjustments eventually develop in the salt-treated adrenalectomized animal which are attended by the restoration of many of the blood values to within normal limits. Similarly, certain peripheral blood effects observed in acute experiments with adrenal cortical extracts and epinephrine are no longer apparent when treatment is continued for longer periods of time. These results are in line with Ingle's (1949) recent comment, based on his findings, that "in

chronic studies of either hormone deficiency or excess, compensatory functions of the organism may partially or completely re-establish homeostasis and thereby mask the effects of the change in hormone intake."

SUMMARY

1. Adrenalectomy in the rat (salt-maintained) results in an anemia which attains its maximum at 2–3 weeks following the operation. Reticulocytosis and decreased red cell fragility accompany the anemic state. The effects diminish after the 3rd week and the values approach approximately normal levels by 60 days.

2. Adrenal cortical extracts, epinephrine, and Compound E prevent the occurrence of these erythrocytic changes. Compound A and DOCA, given to adrenalectomized rats, and adrenal demedullation are without significant influence upon the peripheral blood picture.

3. The peripheral eosinopenia, lymphopenia and neutrophilia produced by daily injections of Compound E (3 mg.) are maintained for at least 14 days.

4. The bone marrow myelograms of adrenalectomized rats show increased granulocytic and diminished nucleated erythrocytic percentages. Adrenal cortical extract concentrates and Compound E (3 mg.) prevent most of these marrow alterations. Reductions in eosinophilic cell percentages and increased vacuolization within the bone marrow are also produced by Compound E. Epinephrine and DOCA increase the percentages of mature myeloid cells and augment slightly the concentration of nucleated erythroid elements in the marrow.

5. Changes in the bone marrow induced by some of the endocrine procedures employed are not associated invariably with altered peripheral blood values; also results obtained after chronic treatment with hormonal factors do not parallel, in all cases, those seen in the acute experiment.

6. The experiments suggest that the adrenal cortex plays a regulative or supportive role in blood-forming processes.

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AN IMMEDIATE KIDNEY RESPONSE TO ACUTE STRESS

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INTRODUCTION

LITTLE is known about non-specific changes in the kidney structure of animals exposed to acute stress. Selye (1937) frequently observed cloudy swelling in the renal tubules of rats during the alarm reaction. Leduc and Guillemin (1949) reported that while the dry weight of most organs is diminished during acute cold exposure, that of the kidney, the heart and the brain remains constant.

In the course of a nutritional study of the alarm reaction we observed that the kidney, like the adrenal, reacts to every type of acute stress with an immediate rise of its weight (Constantinides 1950). The increment becomes measurable as rapidly as 20 hours after commencement of stress but it is of a small order of magnitude.

The data of the present paper show that a renal enlargement is an integral part of the alarm reaction, that it is a function of the adrenal enlargement and that it is primarily due to a hyperplasia of the cells of the proximal convoluted tubules.

MATERIALS AND METHODS

Data from five experiments are presented in this paper.

Experiments No. 1–3 examined the influence of acute stress on the kidney. Formalin injections, cold and forced exercise were the types of stress employed.

Experiment No. 4 investigated the effect of dietary protein on the kidney.

Experiment No. 5 examined the effect of adrenalectomy on the kidney response to cold.

Male rats of the Long-Evans strain, weighing an average of 140 gm., were used in experiments No. 1–4 which were done in Montreal. Male rats of the Sherman strain, weighing an average of 200 gm. were employed in experiment No. 5 which was done in Vancouver.

Experiment No. 1: Formalin injections. A lot of 18 rats was divided in two equal groups. The animals of one group served as untreated controls, while those of the other were given six subcutaneous injections of 0.4 ml. of a 10% aqueous formalin solution, suitably spread over 40 hours. All animals were killed by decapitation three hours after

Received for publication June 1, 1951.

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TABLE 1. SYNTHETIC DIETS USED FOR GAVAGE

(A) *5% Amigen diet*

Amigen[1]	5 gm.
Corn Starch	66 gm.
Sucrose	20 gm.
Mineral Fat (Crisco)	1 gm.
Cod liver oil	1 gm.
Cellu flour	3 gm.
Steenbock mineral mixture[2]	4 gm.
Total solids	100 gm.

To the above solids, 10 ml. of vitamin mixture[3] and tap water up to a total volume of 143 ml. are added, while mixing in a Waring blender.

(B) *25% Amigen diet*

Amigen	25 gm.
Corn Starch	66 gm.

The remaining constituents are exactly the same in type and amount as those of the 5% Amigen diet, except for sucrose which is omitted.

(C) *40% Amigen diet*

Amigen	40 gm.
Corn Starch	51 gm.

The remaining constituents as in diet (B).
N.B. To the solids of diets (B) and (C) vitamin mixture and tap water are added in exactly the same way as with diet (A)
All three above diets contain 2.7 Calories per ml. and are 70% suspensions of solids in water.

[1] "Amigen" = a dried pancreatic hydrolysate of casein containing amino acids and polypeptides, supplied by Mead Johnson & Co., Evansville, Ind.
[2] Steenbock and Nelson: J. Biol. Chem. 56: 355, 1923.
[3] A threefold concentration of the basal, adequate vitamin mixture described by Hawk Oser and Summerson in "Practical Physiological Chemistry," Blakiston Co., 12th edition, p. 1054 (1947).

the last injection. Both adrenals and the left kidney of every rat were fixed in Bouin's fluid and weighed after fixation.

Starting 10 days before and throughout the injection period, the animals of both groups were maintained on a synthetic 25% Amigen diet (table 1, B) administered twice daily by stomach tube. They had free access to tap water.

The 25% Amigen diet has been used by the author as a basal regime in many gavage experiments and it has been proven adequate in all respects as the animals thus fed grow and remain in excellent health. Its protein content should be considered medium.

Experiment No. 2: Exposure to cold. A lot of 12 rats was divided in two equal groups. The animals of one group served as untreated controls while those of the other were exposed to severe cold by being placed (one per cage) in a room with a temperature of 2–4° Centigrade for 24 hours. All animals were killed by decapitation at the end of the cold exposure period. The adrenals and the left kidney of each rat were fixed in Bouin's fluid and weighed after fixation. The nutritional conditions of this experiment were identical with those of experiment No. 1.

Experiment No. 3: Forced exercise. A lot of 12 rats was divided in two equal groups. The animals of one group served as untreated controls while those of the other were forced to run for 20 hours by being placed in drum shaped cages rotating at the rate of 12 cycles/min., with short intervals of rest. All animals were killed and sampled as those of the previous two experiments, at the end of the 20 hour exercise period. The nutri-

tional conditions of this experiment were identical with those of the previous two experiments. Hematoxyline-Eosine sections were prepared from the kidneys of experiments No. 1–3.

Experiment No. 4: Effect of protein on the kidney. A lot of 14 rats was maintained on the basal 25% Amigen diet for 10 days and was subsequently split in two groups:

Group I henceforth received equicaloric amounts of a 5% Amigen diet (table 1, A) a low protein regime.

Group II henceforth received equicaloric amounts of a 40% Amigen diet (table 1, C), a high protein regime.

The animals of both groups were killed by decapitation 48 hours after commencement of the low and high protein regimes. The adrenals and the left kidney of each rat were fixed in Bouin's fluid and weighed after fixation. Hematoxyline-Eosine sections were prepared from the kidneys of this experiment.

Experiment No. 5: Effect of adrenalectomy on the kidney response to stress. Eight normal and 12 adrenalectomized rats were subjected to mild cold by being placed (four to a cage) in a room with a temperature of 6–12° Centigrade for 24 hours. At the end of this period both groups were killed with chloroform and their organs were fixed in Formol-Zenker.

Simultaneously killed and similarly sampled were eight normal and 12 adrenalectomized control rats.

All adrenalectomies were performed 24 hours before commencement of cold exposure.

The animals of this experiment were fed Purina cubes ad lib. and had free access to tap water.

After weighing, paraffin sections from the left kidney of each rat were cut at 5μ and stained with Hemalum-Phloxine-Saffron. All mitoses from metaphase to telophase in random cross sections from each of 6 kidneys per group were counted. To ensure maximum objectivity the counts were done without knowledge of the identity of the samples.

TABLE 2

A) Formalin Injections

	Controls	Formalin injected	% rise	p
Adrenals	19.0 ± 1.1	28.9 ± 1.2	52%	<0.01
Kidney	422 ±10	505 ±16	19.6%	<0.01
No. of animals	9	6		

B) Cold (2–4°C.)

	Controls	Exposed to cold	% rise	p
Adrenals	17.4 ± 0.6	24.0 ± 1.3	40%	<0.01
Kidney	453 ±15	515 ±12	14%	<0.01
No. of animals	6	6		

C) Forced Exercise

	Controls	Exercised	% rise	p
Adrenals	14.5 ± 0.6	18.0 ± 0.8	20%	<0.01
Kidney	318 ± 8	340 ± 7	7%	0.05
No. of animals	6	6		

Effect of stress on adrenals and kidney.

RESULTS

Tables 2–4A contain the means of organ weights expressed as milligrams per 100 grams terminal body weight ± their standard error (ϵ). Table 4B contains the means of mitosis counts of kidney cross sections ± their standard error (ϵ).

a) *Effect of acute stress on the kidney*

As can be seen from table 2, all stressor agents employed caused not only a significant adrenal enlargement but also a significant increase of the kidney weight. It is interesting to note that the reactions of the two organs were of parallel intensity. Under the given experimental conditions, formalin represented the most formidable stress (resulting in a mortality of 3 out of 9 animals) and produced the greatest adrenal and kidney enlargement. On the other hand, the comparatively mild stress of forced exercise resulted in the least adrenal and renal enlargement, while the stress of cold assumed an intermediate position by both criteria.

Routine histological examination of the kidneys from experiments No. 1–3 indicated normal renal structure except for a hypertrophy of the proximal convoluted tubules in the kidneys of the stressed animals.

b) *Effect of dietary protein on the kidney*

The data in table 3 indicate that a change in the protein concentration of the diet from 5% to 40% had no significant effect on the adrenal weight but produced a significant renal enlargement.

Routine histological examination of the kidneys from this experiment revealed normal renal structure except for a hypertrophy of the proximal convoluted tubules in the kidneys of the animals receiving the 40% Amigen diet. It was impossible to distinguish these kidneys from those of the stressed animals. Furthermore, since no mitosis counts were done in either this or the preceding three experiments, the possibility of a concomitant hyperplasia of the proximal convoluted tubules cannot be excluded.

c) *Effect of adrenalectomy on the renal response to cold*

The data in table 4A show that adrenalectomy largely inhibited the renal weight response to cold.

Furthermore, the data in table 4B demonstrate that the kidneys of the stressed animals had a markedly higher mitosis count than those of the non-stressed controls. Adrenalectomy completely abolished the mitosis count rise in response to stress. That there were any mitoses at all in the normal controls was to be expected since these animals were still growing.

Detailed histological study revealed that almost all of the extra mitoses

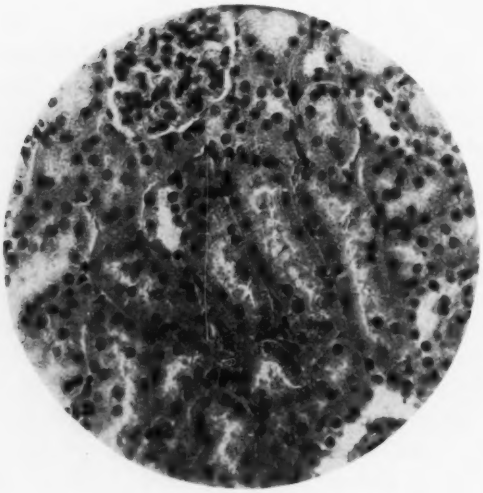

FIG. 1. Normal control kidney. ×260.

which appeared in the kidneys of the stressed intact animals occurred in the cells of the proximal convoluted tubules.

It is noteworthy that the few mitoses in the normal control kidneys were found to be evenly disseminated throughout the cross section of the organ so that the chances of hitting upon a mitotic figure in a random low power field were small (fig. 1). By contrast, the many mitoses in the kidneys of

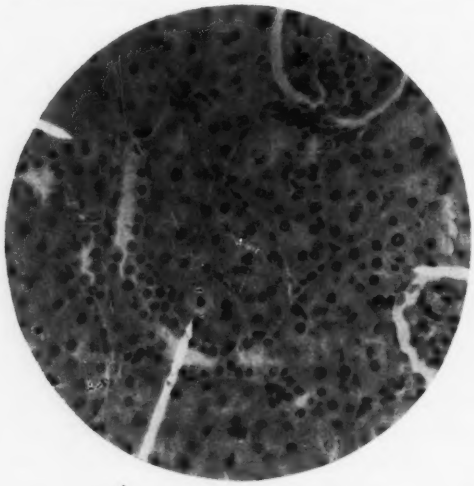

FIG. 2. Kidney of an animal exposed to cold. The white arrows point at mitoses. ×260.

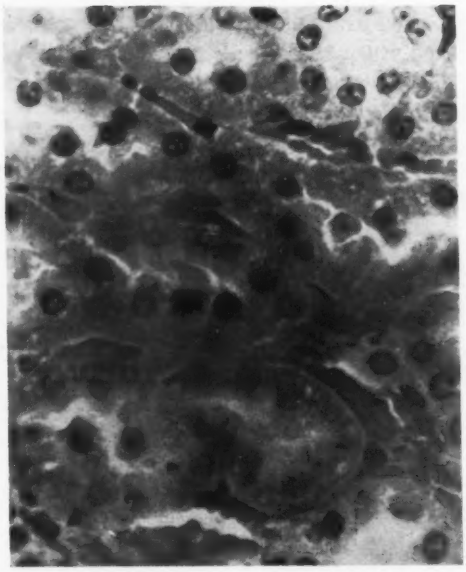

FIG. 3. Kidney of an animal exposed to cold. A metaphase. ×670.

FIG. 4. Kidney of an animal exposed to cold. An anaphase and a metaphase in profile. ×670.

the stressed unoperated animals appeared to be arranged in groups. One such cluster of three mitotic figures is shown in fig. 2.

Figures 3, 4 and 5 show various mitosis stages in the kidneys of stressed rats under high power.

In addition to the above mentioned differences of weight and mitosis count, the cytoplasm of the proximal convoluted tubule cells in the kidneys of the stressed unoperated rats appeared swollen and its basal striation pattern blurred (compare figs. 1 and 2). This qualitative change was too irregular to encourage any attempt of quantitative measurement. It

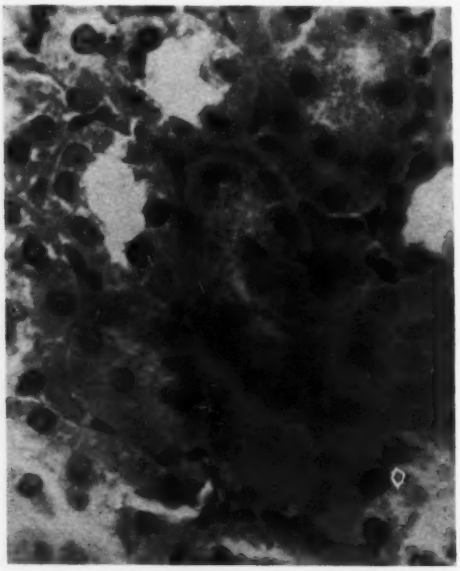

FIG. 5. Kidney of an animal exposed to cold. A telophase. ×670.

probably represents the "cloudy swelling" already described in the literature (cf. Selye 1937).

No edema, hyperemia or other pathological change was visible in any kidney from the four groups of this experiment. The kidneys of the adrenalectomized controls, however, seemed to be somewhat anemic as compared with those of the three other groups.

DISCUSSION AND CONCLUSIONS

It is evident that renal enlargement has to be considered a sensitive morphological criterion of acute stress.

Furthermore, since adrenalectomy largely inhibited this reaction and since plotting the kidney enlargement in the ordinate against the adrenal enlargement in the abscissa yields a straight line (see fig. 6), it appears

that the increment of the renal mass is a linear function of the increment of the adrenal mass, regardless of the nature of stress.

The constancy of the ratio between the increments of the two organs (see table 5) is noteworthy if it is taken into consideration that the values plotted in figure 6 are derived from four self controlled experiments which were different with regard to several experimental variables (rat strain, duration, nature and intensity of stress). It should be noted that experiment No. 5 was done one year after experiments No. 1, 2 and 3.

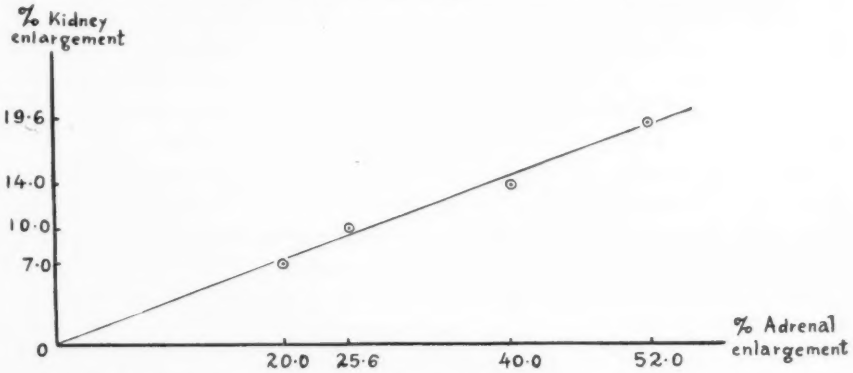

FIG. 6. Graphic correlation of kidney and adrenal enlargements from the four stress experiments of this study.

The question now arises as to the mechanism whereby adrenal activation during the alarm reaction could elicit a renal enlargement.

The data from our dietary experiment indicate that the mere administration of a high protein regime for 48 hours produces a kidney enlargement of comparable magnitude to that elicited by stress of similar duration.

Renal enlargement in response to prolonged protein administration has been frequently described. We need only mention here that Addis et al. (1926) obtained a 20% renal enlargement with "perhaps a hypertrophy of the renal tubules" and no pathological changes in animals fed a 70% protein regime for a year. Similarly Friedman (1948), feeding rats a 60% casein diet for two and a half months, obtained a 25% increase in the kidney weight with histological signs of hypertrophy. On the other hand, kidney enlargement accompanied by some renal damage has been produced with a 75% casein diet administered over a year (Newburgh and Curtis, 1928) or with protracted intraperitoneal protein injections (Baxter and Cotzias). It seems difficult to evaluate these results without adrenal data.

The absence of any significant adrenal reaction to the high protein diet in our experiment would imply that the protein plethora exerts a direct effect on the kidney. In other words, under our experimental conditions, the high protein diet did not constitute a stress in itself.

TABLE 3

	5% Amigen diet	40% Amigen diet	% rise	p
Adrenals	14.1 ±0.7	14.8 ± 0.9	4.9%	0.6
Kidney	292 ±7	355 ±14	21.6%	<0.01
No. of animals	7	7		

Effect of dietary protein on adrenals and kidney.

Admittedly, a direct effect of protein on the kidney mass could be proved conclusively only by its demonstration in adrenalectomized animals. However, even pending such evidence, it is tempting to speculate that the hyperplasia of the proximal convoluted tubules in response to acute stress represents their reaction to the protein catabolism which occurs in the presence of adrenocortical hormones (Ingle 1947, Engel 1951).

Such a hypothesis assumes that the proximal convoluted tubules are in some way concerned with the handling of proteins or protein breakdown products. Oliver's (1948) morphological findings concerning the concentration of exogenous proteins and aminoacids in the cells of the proximal convoluted tubules certainly seem to furnish some evidence in favour of the above assumption.

It is, of course, equally possible that the renal enlargement produced by acute stress is caused by increased blood levels of both protein breakdown products and adrenal steroids. The slight but definite enlargement of the kidney which occurred in the adrenalectomized-stressed rats suggests that

TABLE 4

A) *Adrenal and Renal Weights*

		Controls	Exposed to cold (6–12°C.)	% rise	p
Normal	Adrenals	14.8 ± 0.6	18.6 ± 0.4	25.6%	<0.01
	Kidney	503 ±13	552 ±11	10%	0.01
	No. of animals	8	8		
Adrenal-ectomized	Kidney	481 ± 5	498 ± 5	3.5%	0.02
	No. of animals	12	12		

B) *Mitosis Counts per Kidney Cross Section*

	Controls	Exposed to cold (6–12°C.)	% rise	p
Normal	21 ±6	46 ±6	120%	<0.01
No. of animals sampled	6	6		
Adrenalectomized	9 ±1.6	10 ±1.9	11%	0.7
No. of animals sampled	6	6		

Effect of Adrenalectomy on the Renal Response to Cold.

extra-adrenal factors, perhaps thyroxin (cf. Selye et al., 1945), may be also involved.

Further investigation is therefore needed for a complete analysis of the observed facts.

TABLE 5

	Exercise	Cold (6–12°C.)	Cold (2–4°C.)	Formalin
% Kidney Increment	7.0	10.0	14.0	19.6
% Adrenal Increment	20.0	25.6	40.0	52.0
Ratio $\frac{\%\text{ Kidney Increment}}{\%\text{ Adrenal Increment}}$	$\frac{1}{2.85}$	$\frac{1}{2.56}$	$\frac{1}{2.86}$	$\frac{1}{2.66}$

Kidney/Adrenal Increment Ratio in the four stress experiments of this study.

SUMMARY

1. Acute stress of 20 to 48 hours' duration causes an immediate enlargement of the kidney.

2. The renal enlargement is mainly due to a mitotic proliferation of the cells of the proximal convoluted tubules.

3. Adrenalectomy largely prevents the renal enlargement and completely inhibits the underlying proliferative process.

4. The renal enlargement is a linear function of the concomitant adrenal enlargement.

5. Administration of a high protein regime over 48 hours causes a kidney enlargement of comparable magnitude to that produced by stress, without visibly stimulating the adrenals.

Acknowledgements

A part of this investigation was supported by a research grant from the National Cancer Institute, U.S.P.H.S. The author wishes to thank the Zoology Department, Univ. Brit. Columbia for permission to use a refrigeration room. Thanks are also due to Mrs. M. Phelps for technical help rendered to part of this investigation.

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DEVELOPMENT OF OBESITY IN FORCE FED YOUNG THYROIDECTOMIZED RATS

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RETARDED growth is a well-known effect of thyroid deficiency in the young animal (Simpson, 1913; Salmon, 1938; Scow and Simpson, 1945). Daily food consumption is also subnormal (Persike, 1948) to which the reduced growth rate might be attributed. The present experiments were conducted to ascertain the effect of increased food intake upon the athyroid dwarf. Body weight gain was found to be greater in thyroidectomized rats receiving increased daily rations than in those fed *ad libitum*. The chief chemical composition of their carcass weight gains have been determined and compared with that in normal animals.

METHODS

Thyroparathyroidectomy, or its sham operation, was performed on female Sprague-Dawley rats, 21–27 days old, anesthetized with sodium pentobarbital (intraperitoneal). The thyroidectomized rats are considered to have had total extirpation of the gland, as ascertained in the majority with I^{131} radioautographs of the cervical and mediastinal tissues (Reinhardt, 1942; Scow and Simpson, 1945). Several force fed rats which died before this test could be made are also included since their body composition and other characteristics were similar to those of the I^{131} tested rats.

Rats were divided into five groups: Group A consisted of normal rats fed *ad libitum*; Group B, thyroidectomized rats tube fed an amount nearly equal to that consumed by Group A; Group C, thyroidectomized rats fed *ad libitum;* Group D, normal rats fed one time daily an amount equivalent to that eaten by littermates in Group C; and Group E, normals fed the same amount and in the same manner as Group B. The rats in Groups B and E were fed three times daily at intervals greater than 6 hours beginning with the 1st–3rd postoperative day. A sixth group, Y, of 25-day old female weanling rats was also studied to provide information for estimating initial values for the experimental animals.

A purified type diet was used which consisted of casein, dextrin and cottonseed oil.[1]

Received for publication June 1, 1951.

* Technical assistance by George Carr and William Murphy is gratefully acknowledged.

[1] Diet No. 4185:

Casein (Vitamin free)	18.0
Wesson Oil	8.0
Dextrin	68.8
Salt Mix No. 550 (Osborne & Mendel*)	4.0

* J. Biol. Chem.: **32**: 374. 1917.

A water suspension of this diet, ½ gm. per cc., could be passed easily through an 18-gauge needle providing the casein was sifted through a #80 sieve.[2] Two per cent calcium lactate solution, in addition to drinking water, was always available to each rat.

The rats were sacrificed about 50–60 days postoperative following a 17-hour fast. The carcasses, less the viscera of the neck, thorax and abdomen, were stored frozen in air-tight containers for several months. Water, protein and fat contents of the eviscerated carcasses (including the dissected limbs) were determined according to the procedure of Kochakian *et al.* (1948).

RESULTS

Thyroidectomized rats (Group B) gained weight at an accelerated and continuous rate when the daily food intake, tube fed, exceeded that eaten *ad libitum* (Group C), (figure 1), but there was no significant difference in the nose to anus length (Table 1). Increased thickness of the subcutaneous tissue and marked impingement of the enlarged abdomen and its contents on the chest are vividly illustrated in the roentgenogram in figure 2. Tolerance for the force fed meal did not increase with body weight gain after the midpoint of the experiment which made it impossible to maintain the food intake during the latter half at a level equal to that eaten daily by the *ad libitum* fed normals. Activity of the force fed thyroidectomized rats was markedly reduced in the last two weeks of the experiment, during which time several rats expired spontaneously, without any obvious infection as the cause.

$FeSO_4 \cdot 7H_2O$	.18
$CuSO_4 \cdot 5H_2O$	.02
Vit. A, 55,000 units/gm. Vit. D, 11,000 units/gm.	.03
Thiamine	.0004
Riboflavin	.0006
Nicotinic Acid	.004
Pyridoxin	.0005
Calcium Pantothenate	.004
Choline	.2
Inositol	.02
Biotin	.00002
Folic Acid	.0002
Vit. K	.0002
Vit. E	3 mg./wk. (fed with pipette)

[2] Glucose was unsatisfactory as the source of carbohydrate because of its high osmotic activity in the stomach, potentially fatal in weak animals, and starch, because of its rapid jellification. The suspension was administered through polyethylene plastic tubing attached to an 18 ga. needle and a Luer-Lok syringe. The latter is preferable to glass tip syringes because its metal tip has a larger internal diameter. Expulsion of the food suspension from the syringe was facilitated by occasional rinsings with a mild detergent solution.

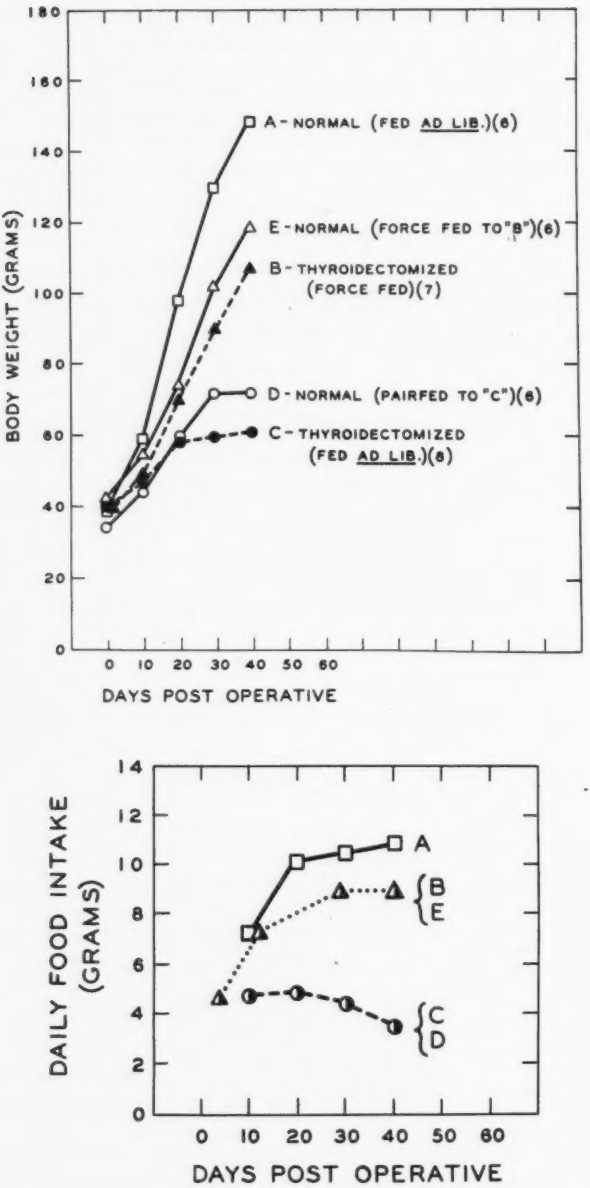

FIG. 1. (a) Body weight gain of thyroidectomized and normal female rats.
(b) Average daily food consumption of thyroidectomized and normal female rats.

The weight gain of thyroidectomized rats fed *ad libitum* (Group C) was markedly reduced around the 21st post-operative day and was associated with a gradual reduction in the daily food intake (figure 1). Both groups of thyroidectomized rats, B and C, gained weight at a slower rate than did their pair-fed controls, Groups E and D, respectively. The subnormal weight gain in the underfed normals, Group D, continued beyond the plateau of the thyroidectomized rats, at 21 days, until the daily food ration

TABLE 1. CARCASS, MUSCLE AND SKELETAL WEIGHTS AND BODY LENGTH OF THYROIDECTOMIZED AND NORMAL FEMALE RATS

Group	No. of rats	Eviscerated carcass		Nose to anus length (cm.)	Forelimb weight[b] (% carcass)	Thigh-calf muscle weight[b] (% carcass)	Femur, tibia, fibula and knee joint wt.[b] (% carcass)
		wt. (gm.)	% live wt.[a]				
85-day old Thyroidectomized							
B—(force fed)	7	83.5 (63–115)	76.5±3.8[c]	15.0±0.8(4)[d]	2.5±0.11	—	—
C—(fed ad lib)	8	44.1 (30–61)	74.8±1.2	14.0±0.2	4.0±0.12	3.5±0.20(5)	1.31±0.09(5)
Normal							
A—(fed ad lib)	6	132.8 (104–125)	81.2±0.9	19.0±0.7	3.4±0.03	6.3±0.08	.95±0.06
E—force fed to "B")	6	106.1 (83–128)	80.2±1.2	18.0±0.4	3.6±0.07	6.9±0.20	1.03±0.03
D—(pair fed to "C")	6	55.7 (51–60)	81.5±0.8	15.5±0.3	4.1±0.11	5.7±0.06	1.32±0.06
25-day old normal							
Y—(weanlings)	6	23.6 (21–25)	73.0±1.2	11.0±0.2	4.4±0.02	4.0±0.09	1.42±0.03

[a] After 17 hr. fast with the exception of Group Y which was not fasted.
[b] Unilateral determination. Forelimb was skinned and separated from carcass along scapular and humeral insertions of thoracic muscles.
[c] Mean ± S.E. (S.E. = $\frac{\text{Range}}{\text{N (number of rats)}}$ (Mantel, 1951)).
[d] Parentheses enclose number of rats measured if less than total number in group.

dropped below 6 gm. per 100 gm. body weight, which was inadequate for growth in small normal rats.

The weight ratio of forelimb to eviscerated carcass was subnormal in the force fed thyroidectomized rats but was normal in thyroidectomized rats fed *ad libitum* (Table 1). The subnormal weight ratio of the thigh-calf muscles to the carcass in the *ad libitum* fed thyroidectomized rats was even less than at the time of thyroid extirpation. Underfeeding normal rats (Group D) did not appreciably alter this muscle-carcass weight ratio. The relative skeletal weight was normal or above normal in the *ad libitum* fed thyroidectomized rats. The combined viscera and mesenteric fat weight, as determined by the weight difference between the eviscerated carcass and the fasted living animal, was relatively greater in both groups of thyroidectomized rats than in the old normals (statistically significant only for Group C).

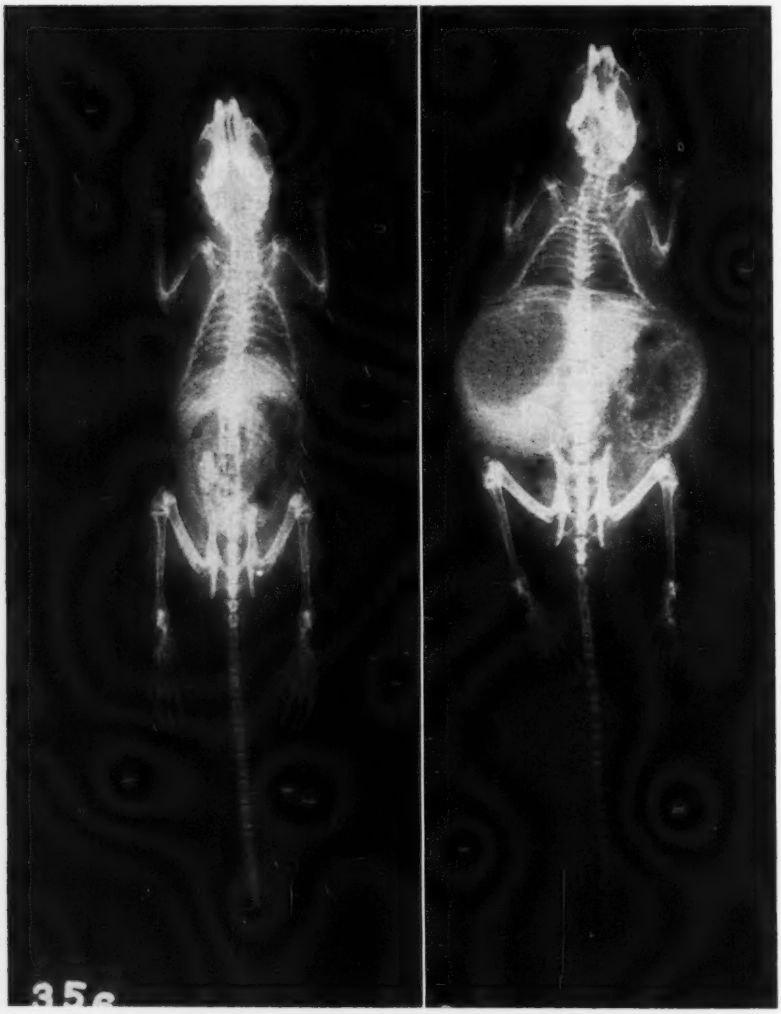

FIG. 2. Roentgenograms of thyroidectomized littermate rats, 55 days post-operative. The rat on the left was force fed 70% more food than eaten *ad libitum* by the rat on the right. Of note is the abundant subcutaneous tissue, the great size of the abdomen and the negligible difference in body length. (1×)

TABLE 2. CHEMICAL COMPOSITION OF EVISCERATED CARCASS OF THYROIDECTOMIZED AND NORMAL FEMALE RATS

Group	No. of rats	Carcass wt. (gm.)	Chemical composition				Water/protein ratio
			% of carcass				
			Water	Protein*	Fat	Fat-free residue†	
85-day old Thyroidectomized B—(force fed)	7	83.5 (63–115)	40.5±2.3‡	12.5±0.5	38.3±2.1	4.9±1.1	3.23±0.14
C—(fed ad lib)	8	44.1 (30–61)	58.6±1.6	17.8±0.7	14.9±2.0	7.0±0.6	3.28±0.10
Normal A—(fed ad lib)	6	132.8 (104–125)	61.5±0.4	20.8±0.3	11.8±0.6	4.9±0.2	2.98±0.02
E—(force fed to "B")	6	106.1 (83–128)	63.4±0.8	20.7±0.4	9.8±0.9	5.1±0.3	3.06±0.04
D—(pair fed to "C")	6	55.7 (51–60)	66.7±0.3	22.1±0.2	5.2±0.5	5.5±0.3	3.02±0.03
25-day old normal Y—weanlings	6	23.6 (21–25)	70.2±0.2	18.5±0.3	6.0±0.4	4.7±0.2	3.79±0.09

* Protein = 6.25 × Nitrogen.

† Fat free residue: This calculation excludes fat not recovered with ether extraction of fatty acids, which is estimated to equal 11% of the weight of the extract.

‡ Mean ± S.E. (S.E. = $\frac{\text{Range}}{\text{N (number of rats)}}$ (Mantel, 1951)).

The final chemical composition of the carcasses is given in Table 2. Of note is the great amount of fat in the force fed thyroidectomized rat. The percent fat in the *ad libitum* fed thyroidectomized rats was also greater than in normals. The water-protein ratio was the same for all 85 day old normals, regardless of the caloric intake. There was no difference in this ratio between the two groups of thyroidectomized rats although there was a 2½ fold difference in carcass fat content.

The chemical nature of the weight gain and the percent retention of ingested food are recorded in Table 3. Storage of fat accounted for 55% of the carcass weight gain in the force fed thyroidectomized rats and was

TABLE 3. CHEMICAL NATURE OF WEIGHT GAIN AND PERCENTAGE OF INGESTED FOOD THAT WAS RETAINED

Group	No. of rats	Carcass gain					Total food intake gm.	Retention (% Amount ingested)		
		Total gm.	Protein		Fat			Protein	Fat	Calories*
			gm.	%	gm.	%				
Thyroidectomized B—(force-fed)	7	55.1	5.20	9.5	30.3	55.1	371	7.8	102.0	19.5
C—(fed ad lib)	8	14.9	2.44	16.4	4.8	32.2	215	6.2	27.9	5.9
Normal A—(fed ad lib)	6	104.4	22.25	21.3	14.0	13.4	471	26.5	37.3	11.1
E—(force fed to "B")	6	75.4	16.33	21.7	8.6	11.4	414	22.0	25.9	8.2
D—(pair fed to "C")	6	30.9	7.71	25.0	1.4	4.5	225	19.0	7.8	4.6

* Calculated on basis of 4.3 calories/gm. food (CHO: 4.1 cal./gm.; Protein: 4.1 cal./gm.; and Fat: 9.3 cal./gm.).

equivalent in amount to the fat ingested. One-third of the weight gain in the slow growing *ad libitum* fed thyroidectomized rats was also fat. Although the total protein gain was greater in those fed larger rations, there was no appreciable difference in the efficiency of protein utilization between the two groups of thyroidless rats. In the normal rats, however, augmentation of the daily ration was accompanied by a marked increase in efficiency of protein utilization. The fat content was also greater in the better nourished normal rats. The greatest percent of ingested calories was retained by the force fed thyroidectomized rats, and the least by the *ad libitum* fed thyroidectomized rats and their pair fed controls.

DISCUSSION

These experiments demonstrate that retardation of growth in thyroidectomized rats is associated with a reduced retention of nitrogen which cannot be overcome by increasing the food intake. Since both the thyroidectomized rat and dog absorb protein at normal rates (Rupp et al., 1949; unpublished data), it is doubtful that the poor nitrogen retention can be attributed to impaired absorption. A continuous body weight gain was produced in thyroidectomized rats by force feeding increased rations daily, but the nature of the weight gain was abnormal since more than half of it was composed of fat. In normal rats, on the other hand, body growth and protein retention were both improved by increasing the daily food intake.

Fat deposited in the carcass of the force fed thyroidectomized rats was equivalent in amount to 102% of the total fat ingested. In view of the facts that the fat analytical procedure used does not recover total fat, that 100% absorption of ingested fat probably did not occur, and that the carcass analyses did not include mesenteric fat depots, it is apparent that the total amount of fat deposited in the body exceeded appreciably the amount that was absorbed. This would then indicate that synthesis of fat occurred in the thyroidectomized rat.

Young hypophysectomized rats also store considerably more fat and less protein than do normal rats when maintained on a food intake adequate for growth in normals (Samuels *et al.*, 1943). These effects are apparently the result of growth hormone deficiency since its administration will increase protein retention and reduce the percent fat in the body (Lee *et al.*, 1934; Li *et al.*, 1948). Part of the dwarfism that develops in thyroidectomized rats has been attributed to growth hormone deficiency (Scow *et al.*, 1949; Koneff *et al.*, 1950). The question is then raised that the excessive accumulation of fat in the young thyroidectomized rat might also be the result of deficiency of growth hormone.

Of considerable interest is the constant ratio between water and protein

content of the carcass for normal rats of similar age which was independent of the wide range of food intake encountered in these experiments but was higher in weanling rats (Table 2). An intermediate level was observed in both groups of thyroidectomized rats which was significantly different from the weanlings and the older normals. Increased water retention related to fat storage was not observed in the obese thyroidectomized rats.

There are several reports that protein retention is equal to 20% of the total body weight gain in normal growing rats and in normal or hypophysectomized rats receiving growth hormone (Young, 1945; Gordon *et al.*, 1948). This ratio was found applicable to the better nourished normal rats in the present experiments but was increased to 25% in those underfed. Its marked reduction in the thyroidectomized rats, to 9.5% in those force fed and 16.4% in those fed *ad libitum*, was compatible with their increased fat retention.

SUMMARY

Impaired nitrogen retention in *ad libitum* fed thyroidectomized rats, associated with reduced rate of growth, was not appreciably affected by a 70% increase in food intake. Increased daily rations, comparable to the above, did increase the nitrogen retention in normal rats.

Force fed thyroidectomized rats continued to gain weight beyond the "growth plateau" typical of those fed *ad libitum*. This growth was abnormal, however, since 55% of the carcass increment was fat and 9% protein. The total amount of fat deposited exceeded that ingested in the diet. The carcass weight gain in *ad libitum* fed thyroidectomized rats was 16% protein and 32% fat, whereas that in normals was 21% protein and 13% fat.

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QUANTITATIVE INHIBITION OF PROGESTERONE BY ESTROGENS IN DEVELOPMENT OF DECIDUOMATA[1]

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IT HAS been known since the observation of Loeb (1907) that the properly sensitized uterine endometrium in laboratory rodents responds to a traumatic stimulus with a rapid local proliferation initiating the maternal portion of the placenta. Since this discovery, many workers have shown that estrogen tends to inhibit the reaction. In a review on estrogen-progesterone interactions on the uterus, Courrier (1950) has performed a commendable task in summarizing the literature to date.

In quantitating the inhibitory action of several estrogens on progesterone in the decidual reaction, we have used the ovariectomized pseudopregnant rat. For this purpose, it was necessary to determine the proper amount of progesterone required to obtain a deciduoma comparable to that elicited in the intact animal. After determining the amount of progesterone required to duplicate a normal response, we proceeded to quantitate the inhibitory effects of six different estrogens on progesterone-induced deciduomata.[2]

MATERIALS AND METHODS

A total of 459 albino virgin rats of an inbred colony derived from a Wistar strain, weighing 200–235 grams, were used in these experiments.

A. *The Normal Decidual Response:* Animals showing fully cornified vaginal smears were selected from the colony and the uterine cervix of each was stimulated by a strong faradic current (Greep and Hisaw, 1938). Vaginal smears were then made daily until the termination of the experiment. Five days after cervical stimulation, the endometrium of one uterine horn of each animal was traumatized by inserting a needle through the tubal sphincter beneath the oviduct down to the cervix. By withdrawing the needle slantwise, the antimesometrial aspect of the endometrium was scratched throughout its entire length. As a result, a decidual response, when present, involved the entire endometrium from cervix to tubal junction. The untraumatized contralateral horn served as a control. The animals were killed 24, 48, and 72 hours post uterine traumatization, and the diameters of both horns of the uterus were measured *in situ*. Decidual reactions

Received for publication June 5, 1951.

[1] This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

[2] We are very grateful to Schering Corporation and Parke, Davis and Company for the generous supply of hormones used in this investigation.

24 hours after traumatization were designated as +2, those at 48 hours as +3, and at 72 hours as +4 (Table 1). These designations were also assigned to deciduomata of comparable diameters when induced in castrated animals by a three day treatment of progesterone (Table 2).

B. *Decidual Reaction in Castrated Rats:* The procedure followed in this group was essentially the same as that described for group "A" with the following exceptions. The animals were ovariectomized at the time of uterine traumatization, and graded doses of progesterone were injected daily for three days. The animals were killed and the uteri measured 72 hours after the first injection of progesterone.

C. *Estrogen-Progesterone Inhibition:* Immediately following castration and uterine traumatization on the fifth day of pseudopregnancy, the various estrogens and a standard dose of progesterone were injected daily for three days. The animals were killed 72 hours after beginning the injections and both horns of the uteri were measured.

All hormones were dissolved in sesame oil, and given in 0.1 cc. per injection.

RESULTS

In an effort to quantitate and correlate the progesterone-induced deciduomata in ovariectomized, pseudopregnant rats with deciduomata in the intact animal, we used a graded dosage of progesterone ranging from one-quarter to two milligrams a day for three days. The information obtained from these experiments is presented in Tables 1 and 2.

TABLE 1. DECIDUOMA FORMATION IN INTACT PSEUDOPREGNANT RATS 24, 48 AND 72 HOURS AFTER UTERINE TRAUMATIZATION

Number of rats	Hours after uterine traumatization	Decidual rating	Mean diameters	
			Traumatized horn	Contralateral horn
			mm.	mm.
11	24	+2	2.9±0.20	1.9±0.17
12	48	+3	3.6±0.34	2.1±0.19
15	72	+4	4.8±0.35	2.2±0.22

TABLE 2. EFFECT OF GRADED AMOUNTS OF PROGESTERONE ON THE DECIDUAL RESPONSE IN OVARIECTOMIZED PSEUDOPREGNANT RATS[1]

Number of rats	Amount of progesterone each day for three days (in mg.)	Decidual rating	Mean diameters	
			Traumatized horn	Contralateral horn
			mm.	mm.
11	0.25	+1	2.4±0.22	1.9±0.20
12	0.50	+2	2.8±0.17	1.8±0.19
11	0.75	+3	3.4±0.23	1.8±0.21
12	1.00	+3	3.5±0.18	1.9±0.20
15	1.25	+3	3.8±0.17	2.0±0.21
16	1.50	+4	4.5±0.25	2.2±0.21
10	1.75	+4	5.1±0.22	2.1±0.23
11	2.00	+4	5.3±0.44	2.2±0.18

[1] Necropsies performed 72 hours after uterine traumatization.

It appears that one-half of a milligram of progesterone a day for three days will elicit the same response found 24 hours after uterine traumatization in the normal pseudopregnant animal. It was found that a very close correlation existed between the measurements of the traumatized and control horns of the uteri of normal and ovariectomized pseudopregnant animals. The uterine horns of the normal animals had diameters of 2.9 mm. and 1.9 mm. for the traumatized and contralateral horns respectively (Table 1), while those of the castrate given one-half of a milligram of progesterone daily for three days measured 2.8 mm. and 1.8 mm. (Table 2). Therefore, this was considered a +2 reaction. Likewise, it was found that 0.75 to 1.25 mg. of progesterone would give a reaction equivalent to a two-day deciduoma, or a +3. The measurements in Tables 1, and 2 for animals given these dosages show a good statistical correlation in that the uteri of injected rats averaged 3.6 mm. and 1.9 mm. while the deciduomata in the intact animals were 3.6 and the control horn 2.1 mm. in diameter.

When the daily dose of progesterone was increased to 1.5 mg. for three days, the deciduomata produced were almost identical with those of normal pseudopregnant rats 72 hours after uterine traumatization. The traumatized horn of those given progesterone had an average diameter of 4.5 mm. and that of the pseudopregnant controls 4.8 mm.; the difference of 0.3 mm. not being significantly greater. If the mean diameters of the deciduomata for the groups given 1.5, 1.75 and 2.0 mg. of progesterone are compared, it will be seen that very little additional decidual growth is produced by doses beyond 1.5 mg. We designated reactions of this magnitude as +4.

For convenience, we assigned a + to reactions where the traumatized uteri measured 2.5 mm. or less. Histological examination of such uteri showed that usually only a very small number of decidual cells were present. Consequently, we considered the + condition as a questionable decidual reaction. Likewise, a 0 was assigned to those uteri in which no decidual cells were found.

Our observations on the decidual response of the uterus to graded doses of progesterone lead us to the conclusion that 1.5 mg. injected daily for three days was a suitable dose for duplicating the +4 reaction seen in the normal animal four days after traumatization of the uterus on the fifth day of pseudopregnancy. Therefore, this dose of progesterone was used as a standard treatment in experiments on the quantitation of the inhibitory action of six different estrogens on decidual development.

Estradiol 17-beta: Our results (Table 3) indicate that of the six estrogens studied estradiol 17-beta is by far the most effective in inhibiting the decidual reaction. Daily injections of 0.08 μg. to 0.110 μg. of estradiol when given concurrently with 1.5 mg. progesterone daily for the three-day period

following uterine traumatization will reduce the decidual reaction to various degrees ranging from +4 to 0. It appears that 0.08 μg. to 0.09 μg. of this estrogen does not modify the reaction, but with each increment of 0.005 μg. there is 25% inhibition. As a consequence of this, 0.110 μg. of

TABLE 3. INFLUENCE OF ESTROGENS ON THE DECIDUAL REACTION INDUCED BY 1.5 MG. PROGESTERONE IN OVARIECTOMIZED PSEUDOPREGNANT RATS*

Estrogens	μg. Estrogen daily dose	Decidual rating	Mean diameters	
			Decidual horn	Contralateral horn
			mm.	mm.
Estradiol 17-beta	0.080	+4	5.1±0.51	2.0±0.22
	0.090	+4	4.7±0.48	2.1±0.20
	0.095	+3	3.8±0.42	2.0±0.21
	0.100	+2	2.8±0.29	2.1±0.19
	0.105	+1	2.2±0.20	2.0±0.19
	0.110	0	1.7±0.18	1.5±0.20
Estrone	0.8	+4	4.9±0.43	2.1±0.22
	1.2	+3	3.7±0.39	1.9±0.15
	1.6	+2	2.9±0.23	2.0±0.13
	2.0	+1	1.8±0.25	1.7±0.24
	2.2	0	1.5±0.20	1.5±0.18
Estriol	5	+4	4.7±0.51	2.1±0.24
	10	+4	4.6±0.44	2.2±0.17
	15	+3	3.7±0.35	2.0±0.15
	20	+2	2.9±0.16	2.1±0.17
	25	+1	2.5±0.12	1.9±0.19
	30	0	2.1±0.14	1.9±0.10
Estradiol benzoate	0.1	+4	4.8±0.39	2.3±0.18
	0.2	+3	3.6±0.24	2.1±0.16
	0.3	+2	2.9±0.17	2.0±0.14
	0.4	+1	2.4±0.18	2.0±0.11
	0.5	0	2.2±0.19	1.8±0.13
Diethylstilbestrol	0.2	+4	4.4±0.24	2.4±0.15
	0.3	+3	3.7±0.27	2.2±0.18
	0.6	+2	2.9±0.20	2.0±0.12
	0.7	+1	2.5±0.19	1.9±0.15
	0.8	0	2.2±0.15	1.9±0.18
Equilenin	5	+4	4.9±0.38	2.4±0.18
	10	+3	3.8±0.21	2.1±0.19
	20	+3	3.6±0.23	2.0±0.20
	30	+2	3.0±0.18	1.9±0.11
	35	+1	2.5±0.20	1.9±0.15
	40	0	2.1±0.13	1.8±0.17

* Each figure represents the arithmetical mean and standard deviation of 5 to 15 animals.

estradiol produces complete inhibition, and the traumatized and contralateral horns are approximately the same diameter.

Estrone: Estrone seems to be approximately 10 to 22 times less effective than estradiol in antagonizing progesterone. It was found that 1.2 μg. of estrone was required to inhibit the reaction 25%, and increments of 0.4 μg.

were necessary to further decrease the reaction to +3, +2, +1, and 0 respectively.

Estriol: It takes considerably more estriol to inhibit the reaction than either estradiol or estrone. Fifteen micrograms appears to be the least amount that will reduce a +4 deciduoma to a +3. A dosage of 20, 25, and 30 μg. will inhibit the reaction progressively from a +3 to 0.

Estradiol benzoate: The benzoate of estradiol is very much weaker than estradiol 17-beta in reducing the decidual reaction. While 0.1 μg. in combination with the standard dose of progesterone does not modify the reaction, an increase of an additional 0.1 μg. reduces the decidual response 25% to a +3, and further increments of 0.1 μg. decreases the reaction stepwise to a negative response.

Diethylstilbestrol: This synthetic estrogen is a rather potent antagonist to progesterone. It only required 0.3 to 0.5 μg to reduce the standard +4 reaction to a +3, while 0.6, 0.7, and 0.8 μg. gave a +2, a +1, and 0 reaction respectively.

Equilenin: This estrogen is an extremely weak antagonist to progesterone. It was found that 10μg. was required to reduce the standard +4 reaction to a +3, and 30, 35, and 40 μg. was necessary for further inhibition to +2, +1, and 0.

These observations make it possible to draw comparisons between the six estrogens used on the basis of their ability to inhibit progesterone under the conditions of these experiments. Several workers have reported estrogen-progesterone (EP) ratios for a number of reactions, including development of deciduomata, in which complete inhibition of progesterone would occur, but, as far as we are aware, none has estimated degrees of inhibition produced by graded doses of estrogen nor have different estrogens been compared in this respect (Courrier, 1950). Therefore, we thought it of interest to calculate the estrogen-progesterone ratio (E:P) in the daily dose at which the minimal amount of estrogen given (1) did not modify the decidual reaction induced by 1.5 mg. of progesterone, (2) the amount of estrogen required for 50% inhibition, and (3) that required for complete inhibition (Table 4).

TABLE 4. ESTROGEN-PROGESTERONE RATIOS (E:P) AND DECIDUAL DEVELOPMENT

Estrogens	E:P that does not modify deciduomata	E:P that inhibits deciduomata 50%	E:P that completely inhibits deciduomata
Estradiol 17-beta	1:16,666	1:15,000	1:13,636
Estrone	1:1,875	1:937	1:682
Estriol	1:150	1:75	1:50
Estradiol benzoate	1:15,000	1:7,500	1:3,000
Diethylstilbestrol	1:7,500	1:2,500	1:1,875
Equilenin	1:300	1:50	1:37

DISCUSSION

Inhibition of the decidual reaction in laboratory rodents by the administration of estrogen has been reported by many workers. Most of these studies have been concerned primarily with the amount of estrogen that would bring about complete inhibition of decidual development, and there has been relatively few instances in which determination of the quantitative relationship of estrogen and progesterone in the inhibitory process was undertaken. While there is considerable published data, comparisons are difficult to make due to differences in hormone preparations and experimental procedures. The literature dealing with this problem has been thoroughtly reviewed recently by Courrier (1950) to which the reader is referred for a more detailed discussion.

The experiments reported here show that the decidual reaction in rats can be used as a sensitive measure for determining the quantitative interaction of estrogen and progesterone. In the first place, decidual development is proportional to the dosage of progesterone (Table 2) and 1.5 mg. daily for three days will produce deciduomata in castrated, pseudopregnant rats by the fourth day that are comparable with those found four days after traumatization of the uterus of the normal rat on the fifth day or pseudopregnancy. When 1.5 mg. progesterone is used as a standard daily dose the amount of estrogen required in graded doses to produce similar degrees of inhibition is characteristic for each of the six compounds tested.

Another point of interest is the observation that the amount of an estrogen required to reduce a +4 reaction to a +3, if used as an increment of dosage, would in each instance produce a further reduction of approximately 25% until complete inhibition was attained. Also, as the dosage of estrogen is increased the point at which inhibition appears seems surprisingly sharp. This is particularly true of the more active estrogens. For example, 0.09 μg. daily of estradiol 17-beta permits a +4 reaction while 0.095 μg. decreases the response to a +3.

When the six estrogens used in these experiments are compared as to their ability to inhibit progesterone in the induction of decidual growth it is found that this capacity is correlated with estrogenic potency, estradiol being the most active and estriol and equilenin the least (Dorfman, 1940). The estrogen-progesterone ratios shown in Table 4 indicate that estradiol is 200 to 300 times more effective as an inhibitor of progesterone than estriol and 300 to 400 times better than equilenin.

Whether or not a given ratio of estrogen and progesterone would produce the same effect when the amount of each hormone was increased was not studied extensively. However, one experiment of this nature with estradiol was of considerable interest. When 0.09 μg. of estradiol was given

daily in combination with 1.5 mg. of progesterone a +4 decidual reaction resulted (E:P ratio 1:16,666). It was found that when this ratio was maintained the dosage could be increased five fold without modifying the reaction (Table 5). This of course may not be true for other ratios or other estrogens but is suggestive and should be pursued further.

It is probably futile to speculate on the nature of this reaction, but the wide difference in the amount of the several estrogens required to inhibit a standard dose of progesterone suggests that the competition may not be one in which two species of molecules compete for the same position in an enzyme system. If this were so, and it is assumed that estrogen and progesterone reach the site of the reaction with equal facility, one would suppose that the advantage would be on the side of the compound represented by the greatest number of molecules. The estrogen-progesterone ratios shown in Table 4 make this appear as an unlikely explanation. It also seems possible that estrogen may be concerned with an entirely different metabolic process from that controlled by progesterone and that the competition is between enzyme systems. There is some evidence that supports this thought, but as yet it is inconclusive.

TABLE 5. INFLUENCE OF INCREASED DOSAGE IN ESTRADIOL: PROGESTERONE RATIOS ON DECIDUAL DEVELOPMENT IN OVARIECTOMIZED PSEUDOPREGNANT RATS*

Estradiol 17-beta daily dose in μg.	Progesterone daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn	Contralateral horn
			mm.	mm.
0.09	1.5	+4	4.7±0.51	2.0±0.22
0.18	3.0	+4	4.6±0.30	2.2±0.18
0.27	4.5	+4	4.8±0.14	1.9±0.17
0.36	6.0	+4	4.5±0.11	2.0±0.12
0.45	7.5	+4	5.8±0.14	2.1±0.15

* Each figure represents the arithmetical mean and standard deviation of 3 to 4 animals.

SUMMARY

Six estrogens were compared as to their ability to inhibit the decidual growth in rats induced by a standard dose of progesterone. The rats were castrated on the fifth day of pseudopregnancy, one horn of the uterus traumatized by scratching the endometrium on the antimesometrial surface throughout its entire length, and each animal was given 1.5 mg. progesterone daily for three days and killed 72 hours after the first injection. Deciduomata produced by this method were approximately of the same diameter as those found in normal animals on the fourth day following uterine traumatization on the fifth day of pseudopregnancy. Each estrogen was tested to determine (1) the minimal daily dose that could be given concurrently with 1.5 mg. progesterone without modifying the decidual re-

action, (2) the amounts that would reduce the reaction 25%, 50%, and 75%, and (3) the minimal dose that would give total inhibition. Estradiol 17-beta was by far the most active inhibitor. The other estrogens listed in order of their effectiveness were estradiol benzoate, diethylstilbestrol, estrone, estriol, and equilenin, the latter two being 200 to 300 and 300 to 400 times less active than estradiol.

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FURTHER OBSERVATIONS ON THE ROLE OF THE ADRENAL CORTEX IN THE METABOLISM OF AMINO ACIDS

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PREVIOUS data derived from the measurement of urea formation in nephrectomized rats have been interpreted to indicate that the adrenal cortex was not concerned in the catabolism of amino acids (Engel, Schiller and Pentz, 1949; Bondy, Engel and Farrar, 1949). Neither adrenal cortical extract nor previous adrenalectomy were found to have any measurable effect on the rate of urea formation during a 3–4 hour period following the intravenous administration of amino acids. Indeed, our results were interpreted as indicating that the intravenous administration of amino acids actually suppressed the catabolism of endogenous protein that occurred after administration of cortical extract. Bondy (1949) showed indisputably that adrenal extract had an effect on peripheral protein by demonstrating an increased accumulation of amino acids in the plasma of eviscerated rats treated with adrenal extract. This has been confirmed in cortisone-treated eviscerated rats (Ingle, 1950). Both the accelerated accumulation of amino acids in the eviscerated rat and the increased urea formation in the nephrectomized rat given adrenal extract could be abolished by the administration of glucose. This glucose effect was likewise interpreted as acting at the level of whole protein in the periphery.

Recently, a report has appeared by Cagan, Gray and Jensen (1950) which is in conflict with the above interpretations. These investigators reported that the amino acid oxidase activity of liver and kidney homogenates from adrenalectomized animals was less than from normals and could be restored to normal either by treatment of the animals with cortical extract or by the addition of extract to the homogenates. These same investigators found that administration of a casein hydrolysate to normal rats caused a striking increase in the amino acid oxidase activity of liver and kidney homogenates, but such an increase did not occur when the same amino acid solution was given to adrenalectomized rats. The latter animals showed a delayed clearance of amino acids from their blood and lesser increases in blood urea and glucose than normal animals, results which were

Received for publication June 5, 1951.

* Aided by a grant from the American Cancer Society, administered by the Committee on Growth of the National Research Council.

interpreted as reflecting impaired deamination in these animals. These results, however, are open to the criticism that the adrenalectomized animals may not have been in good condition and may have been suffering from varying degrees of peripheral circulatory insufficiency. The somewhat elevated blood amino acids and urea and low blood sugars and high hematocrit in the control adrenalectomized animals suggest this. Since both normal and adrenalectomized animals show a delayed clearance of injected amino acids from their blood streams, impaired urea formation and decreased amino acid oxidase activity in their livers during peripheral circulatory failure as well as the blood changes noted above, caution is necessary in interpreting such changes as being related specifically to adrenal insufficiency (Engel, Winton, Long, 1943; Wilhelmi, Russell, Engel and Long, 1945; Engel and Engel, 1946). Furthermore, we have found the intraperitoneal injection of Amigen to be an alarming and often shock-inducing procedure, particularly in the adrenalectomized animal. The increase in hematocrit in Cagan *et al.*'s experiment strongly suggests that their animals were in circulatory failure. The increased hematocrit could also account for the increase in whole blood amino nitrogen since the concentration of amino acids in red cells of rats is considerably higher than in plasma.

Our own results described above may likewise be criticized on the grounds that the period of measurement of urea formation after amino acid injection was too long and hence any delay in deamination occurring in the adrenalectomized animal may not have been detectable. In the normal animal urea formation from a single injection of amino acid is generally completed within 2 hours (Engel and Engel, 1946).

For this reason the rates of urea formation in nephrectomized rats with and without adrenal glands and in animals treated with varying doses of ACTH were reinvestigated. Rates were measured at hourly intervals after intravenous injection of amino acids with and without glucose and, at the same time blood amino nitrogen was measured. In addition observations on the effects of glucose on deamination and urea formation were made.

MATERIALS AND METHODS

Male rats of the Vanderbilt strain, weighing 220–280 grams were used. Except where otherwise indicated the animals were fed a synthetic diet low in potassium (Bondy and Engel, 1948) for four days prior to nephrectomy or combined adrenalectomy-nephrectomy. This was done to allow for longer survival of adrenalectomized rats after npehrectomy. Adrenalectomized rats were given 0.5 mg. desoxycorticosterone acetate subcutaneously after adrenalectomy. Adrenalectomy and nephrectomy were performed as a single operation approximately 18 hours before the test period. Blood urea was determined by the method of Archibald *et al.* (1945), blood amino nitrogen by the method of Frame *et al.* (1943) and blood sugar by the method of Somogyi (1945). The method of calcula-

tion of urea formation as mg. urea N/100 grams body weight per hour was as in the previous report (Engel, Schiller and Pentz, 1949).

RESULTS

The effects of a single intravenous injection of 1.0 ml./100 grams body weight of a 10% mixture of amino acids (Merck's Amino Acids Solution)

TABLE 1. UREA FORMATION FROM AMINO ACIDS WITH AND WITHOUT GLUCOSE. ADRENALECTOMY AND ACTH

Group	No. of rats	Urea as mg. per 100 gms. body weight per hour					
		0–4 hrs.	4–5 hrs.	Change	5–6 hrs.	Change	Total Urea N
Control—Amino Acids*	27	1.96±0.07	7.09±0.27	+5.12±0.28	4.96±0.31	+3.00±0.32	8.14±0.45
Control—Amino Acids in 20% Glucose	28	2.06±0.08	5.90±0.34	+3.56±0.46	4.02±0.36	+2.00±0.37	5.56± .48
Control—20% Glucose	12	2.13±0.15	2.13±0.15	0	2.50±0.27	+0.37±0.27	0.37±0.42
Adrx—Amino Acids	15	1.12±0.09	4.91±0.31	+3.79±0.31	4.37±0.33	+3.24±0.36	7.03±0.46
Adrx—Amino Acids in 20% Glucose	14	1.40±0.13	3.16±0.46	+1.76±0.49	3.56±0.38	+2.15±0.31†	4.31±0.64
Adrx—20% Glucose	14	1.25±0.11	1.76±0.22	+0.51±0.22	1.53±0.25	+0.28±0.25	0.79±0.33
Control—20% Glucose	10	1.73±0.08	1.52±0.26	−0.21±0.24	1.98±0.42	+0.25±0.41	+0.04±0.40
4 mg. ACTH—Amino Acids	17	2.31±0.07	7.84±0.41	+5.53±0.39	4.26±0.42	+1.95±0.44	7.48±0.44
4 mg. ACTH—Amino Acids in 20% Glucose	17	2.30±0.10	7.50±0.42	+5.20±0.46	4.32±0.47	+2.02±0.49	7.22±0.77
4 mg. ACTH—20% Glucose	18	2.10±0.10	1.66±0.19	−0.44±0.20	1.87±0.15	−0.23±0.19	−0.67±0.29
8 mg. ACTH—Amino Acids	13	2.36±0.12	7.63±0.52	+5.27±0.52	4.11±0.33	+1.81±0.31	+7.08±0.59
8 mg. ACTH—Amino Acids in 20% Glucose	16	2.39±0.08	6.58±0.44	+4.60±0.32	4.60±0.32	+2.21±0.31	+6.81±0.48
8 mg. ACTH—20% Glucose	13	2.31±0.09	2.09±0.30	−0.22±0.22	1.67±0.28	−0.64±0.40	−0.86±0.18

* Amino Acids Solution Merck injected in a dose of 1 ml./100 gms. body weight (13.3 mg. N).
† 11 observations.

TABLE 2. EFFECT OF ADRENALECTOMY AND ACTH ON BLOOD AMINO N AFTER INTRAVENOUS AMINO ACIDS WITH AND WITHOUT GLUCOSE

Group	No. of rats	Whole blood amino nitrogen, mg. per 100 ml.				
		4th hour	5th hour	Change	6th hour	Change
Control—Amino Acids	27	11.6±0.24	17.3±0.43	+5.7±0.31	13.0±0.25	+1.4±0.13
Control—Amino Acids in 20% Glucose	28	11.8±0.23	17.5±0.42	+5.7±0.41	12.5±0.22	+0.7±0.28
Control—20% Glucose	12	11.2±0.21	11.3±0.30	+0.1±0.21	10.6±0.30	−0.6±0.14
Adrx—Amino Acids	15	10.6±0.26	17.3±0.45	+6.7±0.42	11.9±0.27	+1.3±0.21
Adrx—Amino Acids in 20% Glucose	15	11.3±0.31	20.5±0.70	+9.2±0.62	15.3±0.76	+3.3±0.69
Adrx—20% Glucose	13	10.1±0.30	10.3±0.31	+0.2±0.18	9.6±0.29	−0.5±0.19
Control—20% Glucose	10	11.3±0.24	11.7±0.29	+0.4±0.29	11.3±0.23	0
4 mg. ACTH—Amino Acids	17	12.2±0.26	17.0±0.49	+4.8±0.42	13.3±0.31	+1.1±0.28
4 mg. ACTH—Amino Acids in 20% Glucose	17	12.3±0.20	17.2±0.42	+4.9±0.31	13.0±0.23	+0.7±0.16
4 mg. ACTH—20% Glucose	18	12.0±0.23	12.0±0.24	0	11.7±0.26	−0.3±0.10
8 mg. ACTH—Amino Acids	14	12.5±0.22	18.1±0.41	+5.6±0.42	13.7±0.28	+1.2±0.22
8 mg. ACTH—Amino Acids in 20% Glucose	17	12.3±0.25	16.6±0.42	+4.3±0.52	12.7±0.19	+0.4±0.23
8 mg. ACTH—20% Glucose	13	11.6±0.25	11.6±0.25	0	11.4±0.17	+0.2±0.15

on urea formation and on blood amino nitrogen levels are shown in Tables 1 and 2. One ml. of the amino acid mixture contained 13.3 mg. nitrogen. After a four hour control period the amino acid mixture was injected into

the external saphenous vein of the rats under light nembutal anesthesia and blood samples taken again at exactly one and two hours after injection. Examination of the control data of the first four hours shown in the third column of Table 1 reveals that the rate of urea formation was significantly lower in the adrenalectomized animals compared to either the untreated controls or those receiving ACTH. The ACTH treated animals which received 1 or 2 mg. of Armour's ACTH at approximately six hour intervals for 24 hours before the injection of amino acids had basal rates of urea formation only slightly greater than the controls and there was no difference between the responses to the two doses of ACTH. The difference between the controls and ACTH treated rats is statistically significant if all the control data and the ACTH data respectively are pooled. Since the four control groups and the six ACTH groups were each treated identically up to the fourth hour, this is a valid comparison. The increase in urea formed after injection of amino acids is shown for one hour in the fifth column, for two hours in the seventh column and the total in the eighth column. Inspection of the data in the fifth, seventh and eighth columns reveals the following significant findings: in control animals the amount of urea formed during the first hour after intravenous amino acid injection was significantly less when the amino acids were injected with 20% glucose. Since this same amount of glucose alone did not decrease urea formation when injected intravenously (Glucose Control) the decrease in urea formation must represent an inhibition of deamination of the injected amino acids. This was not reflected in any difference in the one hour post injection blood amino nitrogen levels (Table 2). During the second hour the urea formed was also somewhat less, but not highly significantly so, while the total two hour urea accumulation was highly significantly depressed in the glucose treated animals. The adrenalectomized animals given amino acids alone likewise showed a significant depression in urea formation during the first hour, but this was made up in the second hour so that the total amount of urea formed was not significantly different from the controls, confirming previous results (Bondy, Engle and Farrar, 1949). The blood amino acid increase was insignificantly greater at the first hour and identical with the control in the second hour (Table 2). In contrast, the adrenalectomized rats given amino acids in 20% glucose showed a striking depression in urea formation during the first hour compared to all other groups receiving amino acids with or without glucose and a depression in urea formation in the second hour compared to both controls and adrenalectomized receiving amino acids alone, but not compared to others receiving amino acids and glucose. Nevertheless the total urea formed in two hours was highly significantly less in the adrenalectomized rats receiving amino acids in glucose than all other groups. The depression in deamination was

reflected in the significant elevations in blood amino acids at both the fifth and sixth hours in this group (Table 2). Twenty per cent glucose did not significantly alter urea formation in the adrenalectomized rats. The ACTH treated animals at both dose levels of ACTH showed identical rates of urea formation after amino acids regardless of whether glucose was given or not and these rates were the same as in the controls given amino acids alone, again confirming earlier results. On the other hand, the first hour rates and total urea in the ACTH groups receiving amino acids in glucose were significantly greater than in the corresponding control and adrenalectomized groups receiving amino acids in glucose. In short, glucose did not exert an inhibitory effect on amino acid deamination in the ACTH treated animals. Glucose, however, did depress slightly the rate of urea formation from endogenous amino acids in the two ACTH treated groups receiving 20 per cent glucose alone again confirming earlier results (Engel, Schiller and Pentz, 1949).

Whole blood amino nitrogen levels (Table 2) prior to amino acid injection were lower in two of the adrenalectomized groups than either controls or ACTH treated animals, but these differences are of questionable significance. The pooled value for whole blood amino nitrogen in the ACTH treated rats is, however, significantly higher than that for the adrenalectomized rats. Only the fifth and sixth hour increases in amino nitrogen in the adrenalectomized animals given amino acids and glucose were highly significant statistically compared to all other groups receiving amino acids with or without glucose. Except for this one group there was no evidence from the amino nitrogen data that adrenalectomized rats were less able to clear injected amino acids from their blood or that ACTH treatment modified the rate of clearance of amino acids. With the exception of the last group receiving 8 mg. ACTH and glucose all groups injected with 20% glucose showed a small, but significant decrease in blood amino nitrogen during the sixth hour. This may reflect hemodilution.

It is of interest that the amount of glucose used in these experiments, i.e. 200 mg./100 grams body weight did not decrease the basal rate of urea formation except in those animals receiving ACTH. On the basis of previous work (Engel, Schiller and Pentz, 1949; Bondy, 1949) this small decrease represents a depression of endogenous protein catabolism. This makes the increase in urea formation in the ACTH treated rats given amino acids in glucose compared to similarly treated controls of greater significance. However, if larger amounts of glucose are given to either control or adrenalectomized rats, a significant suppression in the basal rate of urea formation may be demonstrated. This presumably represents inhibition of both endogenous protein catabolism and amino acid deamination. Results of such an experiment are seen in Table 3. Here, after a four hour

control period, 0.5 ml. of 50% glucose per 100 grams was given by stomach tube, and repeated in two hours, a total of 500 mg. of glucose/100 gms. In both the control and the adrenalectomized animals the decrease in urea formation during glucose treatment was significant, amounting to 56% in the adrenalectomized rats and 34% in the controls.

DISCUSSION

The results of these experiments in which amino acids were injected intravenously into adrenalectomized and ACTH treated nephrectomized rats are in substantial agreement with earlier results (Engel, Schiller and

TABLE 3. EFFECTS OF GLUCOSE ON UREA FORMATION IN NEPHRECTOMIZED AND NEPHRECTOMIZED-ADRENALECTOMIZED RATS

Group	No. of rats	Urea as mg. N per 100 gms. body wt./hour			Blood sugar—mg. per cent			
		0-4 hrs.	4-8 hrs.	Change	0 hr.	4th hr.	6th hr.	8th hr.
Nephrectomy	10	1.62±0.08	1.12±0.06	−0.55±0.12	—	120±3.6	158± 5.8	164± 8.5
Nephrectomy-Adrenalectomy	10	1.21±0.13	0.53±0.12	−0.68±0.15	74±3.8	68±1.5	131±13.6	110±12.0

All rats received 0.5 ml. of 50% glucose/100 gms. by stomach tube at the 4th and 6th hours.

Pentz, 1949; Bondy, Engel and Farrar, 1949) in that the total amount of urea formed in two hours was not significantly different from that in control rats. However, when the rates are broken down into hourly periods is it seen that a significant delay in deamination is apparent during the first hour after injection of amino acids, but this is largely made up during the second hour. It is conceivable that this is a reflection of a slower clearance of amino acids by the liver on a circulatory basis, without, however, there being any evidence of peripheral circulatory failure in these experiments. Since there was no corresponding increase in deamination in the ACTH treated rats after amino acids, this experiment leaves unsolved the question whether there is an intrinsic defect in hepatic deamination which is independent of circulatory alterations in the adrenalectomized animal. The lack of difference in plasma amino nitrogen between the control and adrenalectomized animals fails to confirm the results of Cagan *et al.* (1950).

However, the results following injection of amino acids in 20% glucose reveal a clear-cut effect of glucose and of the adrenal cortex on deamination of amino acids. In contrast to earlier experiences in which 50 mg. of glucose per 100 grams injected with amino acids had no apparent effect on deamination, 1 ml. of 20% glucose per 100 grams effectively depressed amino acid deamination. This occurred in animals with intact adrenals and even more strikingly in adrenalectomized rats, but not in those treated with ACTH. This marked suppression of urea formation from amino acids in

the adrenalectomized animals and lack of effect of glucose in the ACTH treated animals establishes an influence of the adrenal cortex on amino acid deamination which is probably independent of circulatory factors. In the adrenalectomized animals the decreased urea formation was paralleled by a substantial delay in clearance of blood amino acids. Physiologically, this finding is of considerable significance since the effect on deamination occurs under circumstances which more closely correspond to those which the organism ordinarily must deal with, i.e. the simultaneous presentation of amino acids and carbohydrate to the liver for metabolism.

Thus there have now been demonstrated two effects of glucose on the metabolism of protein and amino acids in relation to the adrenal. It is altogether probable that both are operative at the same time even though not necessarily demonstrable by the method used in this study. Small doses of glucose (50 mg. per 100 grams) will effectively suppress the extra urea formed following the injection of ACTH, adrenal extract or following nonspecific stress but will not cause a measurable inhibition of urea formation from injected amino acids (Engel, Schiller and Pentz, 1949; Engel, 1951). The latter has also been true in both adrenalectomized-nephrectomized and nephrectomized rats when urea formation was measured over a one hour period after amino acid injection. Thus in one such experiment nephrectomized animals formed 11.9 ± 1.3 mg. urea N/100 grams in one hour after intravenous amino acids and 12.1 ± 1.1 mg. urea N/100 grams in one hour after the same dose of amino acids in 5% glucose. Adrenalectomized animals formed 13.7 ± 1.1 and 13.3 ± 1.3 mg. urea N/100 grams respectively after the same amino acid solutions. None of these values are statistically significantly different one from another. The 50 mg. of glucose only has a measurable effect on the extra urea formation which has its origin in the added endogenous protein catabolism arising from the above stimuli. A larger dose of glucose, 200 mg. per 100 grams has a readily demonstrable effect in suppressing extra urea formation from injected amino acids, but has no measurable effect on basal endogenous protein breakdown as reflected in the basal rate of urea formation. And finally when a dose of 500 mg. of glucose per 100 grams is employed, the basal rate of urea formation is depressed in both adrenalectomized animals as well as those with intact glands. This probably represents a combined effect in decreasing protein catabolism (and/or increasing protein anabolism) as well as decreasing amino acid deamination. Regrettably plasma amino nitrogen levels were not measured in the animals given 500 mg. of glucose. However, it is of some interest that Friedberg and Greenberg (1947) have reported subsubstantial elevations in the plasma amino acid levels of animals fed protein free diets for several weeks.

The mechanism by which glucose decreases urea formation and the role

of the adrenal cortex in this process remains unexplained. Carbohydrate has a well-known "sparing" effect on protein which undoubtedly involves favoring protein synthesis as well as inhibiting protein breakdown. Some of the decreased urea formation after glucose could represent increased utilization of amino acids for protein synthesis, an interpretation supported in the animals with intact adrenals by the fact that the blood amino acids following amino acid-glucose injection were the same as those in the rats that formed more urea when amino acids were given without glucose. In the adrenalectomized animals, however, there was an increase in blood amino acids and glucose so that increased protein synthesis cannot be the sole explanation of the lessened urea formation. Nevertheless, it is of interest that Hoberman (1950) using N^{15} glycine as a tracer did adduce evidence that the adrenalectomized animal does have a greater rate of protein synthesis than corresponding controls. One might anticipate that in the adrenalectomized animal which has a low rate of gluconeogenesis and a comparatively high rate of glucose utilization including conversion of carbohydrate to fat (Welt and Wilhelmi, 1950) the available carbohydrate supplies at any moment might be the limiting factor in determining the rates of protein synthesis. In the presence of a plethora of carbohydrate, protein synthesis may proceed at a maximal rate. In animals getting ACTH the presumed inhibition of carbohydrate utilization might make available less energy for protein synthesis.

Although protein anabolism may be a factor in these results, the high blood amino acids in the adrenalectomized rats receiving amino acids and glucose suggest that a decreased rate of deamination is a more significant influence. Other than brief mention by Hoberman and Graff (1950) of some unpublished studies we know of no previous data demonstrating that glucose inhibits amino acid catabolism. If one assumes that, under ordinary circumstances in the fasting animals, products of amino acid catabolism serve both as a source of new carbohydrate as well as of certain Krebs cycle intermediaries, notably oxalacetic acid, the observation that the administration of glucose suppresses urea formation from injected amino acids is not a surprising one. The finding that a clear cut modification in urea formation from injected amino acids occurs in the adrenalectomized or ACTH treated rats only in the presence of a plethora of glucose throws emphasis again on the concept that the central locus of action of the adrenal cortex is at some step in the utilization of carbohydrate, possibly in the Krebs cycle, and that all other changes are secondary. In the 18 hour fasted adrenalectomized animal carbohydrate stores are depleted and the blood sugar falling but the amount of carbohydrate available for utilization at the time of amino acid administration is probably not greatly different from normal and hence the amino acids are utilized almost as

well as normal. Similarly, the difference in carbohydrate utilization between the fasted normal and the fasted ACTH treated animals may not be sufficiently great to influence the fate of the injected amino acids. On the other hand, in the presence of a glucose load the glucose may be so much more the preferential source of Krebs cycle intermediaries in the adrenalectomized animal in which little carbohydrate is stored as glycogen, that amino acid deamination is held in abeyance. In the ACTH treated animal injected with glucose and amino acids more glucose is stored as glycogen and presumably less used by oxidation and conversion to fat. Hence injected glucose has little inhibitory effect on deamination.

In the above experiments great care was used to avoid the complications of peripheral circulatory failure in the adrenalectomized animals. As indicated earlier, a failing circulation has very striking effects on hepatic metabolism of amino acids. Clinically all our rats were in good condition as indicated by their appearance and ease of bleeding. Blood amino nitrogen levels in the adrenalectomized animals were, if anything, slightly lower than normal indicating that there was no hemoconcentration and very likely no circulatory failure. It might be mentioned that although blood amino nitrogen levels are recorded only for the fourth hour, in many animals they were also measured at the beginning of the experiment, but never found to be significantly different. A rising blood amino nitrogen level has been found to be a sensitive indication of peripheral circulatory collapse (Engle, Winton and Long, 1943). It is our impression that such differences as there are between the results described herein and those of Cagan *et al.* are due to the presence of varying degrees of peripheral circulatory failure in the latters' animals.

SUMMARY

1. The basal rate of urea formation 18 hours post-operatively was significantly greater in ACTH treated nephrectomized rats than in control nephrectomized rats. The latter, in turn, had a significantly higher rate than adrenalectomized-nephrectomized rats. 200 mg. of glucose/100 gms. body weight have no effect on the basal rate of urea formation in the latter two groups but reduced that of the ACTH treated rats to the control level.

2. Five hundred milligrams of glucose/100 grams body weight significantly decreased the basal rate of urea formation in nephrectomized and adrenalectomized-nephrectomized rats.

3. The hourly rate of urea formation after an intravenous injection of amino acids was significantly decreased during the first but not the second hour after injection in adrenalectomized-nephrectomized rats compared to control or ACTH treated nephrectomized rats which did not differ one from the other in their rates of urea formation after injected amino

acids. The slightly delayed urea formation after amino acid injection in the adrenalectomized-nephrectomized rats was not reflected in a delayed fall in blood amino nitrogen.

4. Two hundred milligrams of glucose per 100 grams body weight significantly decreased the amount of urea formed one and two hours after amino acid injection in control nephrectomized and adrenalectomized-nephrectomized rats but not in ACTH treated nephrectomized rats. Blood amino nitrogen one and two hours after amino acid injection was significantly higher in the glucose treated adrenalectomized-nephrectomized rats receiving amino acids than in the correspondingly treated control and ACTH treated nephrectomized rats.

5. It is concluded that the adrenal cortex exerts an influence on amino acid catabolism in the liver as well as on protein catabolism in the periphery but that the former is only apparent by the techniques used here in the presence of a plethora of glucose.

ACKNOWLEDGEMENTS

We are indebted to Mrs. Rosalie Green for technical assistance; to Dr. A. Gibson of Merck and Company for generous supplies of Amino Acids Solution and to Dr. J. Mote of Armour and Company for ACTH.

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THE RESPONSE OF THE THYROID GLAND OF THE RAT TO SEVERE STRESS

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STRESSING agents produce a decrease in the activity of the thyroid gland. Williams *et al.* (1949) found that the I^{131} uptake in the thyroid was decreased by the administration of adrenaline or typhoid vaccine or by trauma. A similar result was obtained with other stressing agents: formalin injections (Paschkis *et al.* 1950), anoxia, starvation, and vitamin deficiencies (Van Middlesworth and Berry, 1950), and adrenaline injections (Soffer *et al.*, 1950).

Recently, Money *et al.* (1950), studying the effects of adrenal cortical products on the uptake of radioiodine in the thyroid of the rat, found that ACTH and cortisone greatly decreased the uptake. These results were confirmed by Reiss (1950) at least in normal human subjects.

This is an attempt to study further the effect of stressing agents on the thyroid gland of the rat using both a chemical method for the determination of the uptake of radioiodine and a radioautographic method for the vizualization of the sites of the uptake.

MATERIALS AND METHODS

A total of 111 male hooded rats weighing between 125 and 150 grams were used. These animals had been fed on a diet of Purina Fox Chow. Because animals under various stresses do not necessarily consume the same quantity of food as do completely normal animals, all except one group of rats in the first two experiments were given no food for the duration of that experiment. Control of the food intake also provided a control of the iodine intake.

In each of the experiments the animals were divided into groups of 5 to 9 animals. These were then subjected to the stressing stimuli during twenty-four hours prior to injection of radioiodine (I^{131}). The types of stress to which different groups of rats were submitted were:

1) Forced muscular exercise, in a revolving drum for 24 hours with several intervals of rest;
2) 5 subcutaneous injections of 10% formalin throughout the day, the first injection containing 0.5 cc. of solution, the subsequent injections containing 0.3 cc. of solution; and
3) Transection of the spinal cord performed at the level of the 7th cervical vertebra.

Each animal received a single subcutaneous injection of tracer radioiodine in the form of sodium iodide containing 25μc. in a volume of 0.1 cc. An intravenous route of injection (jugular) was taken in Experiment 2, however. In Experiment 4, 1 mg. of non-radioactive iodide (in the form of potassium iodide) was added to each injection dose.

Received for publication June 9, 1951.

The animals were sacrificed under anesthesia two hours after the radioiodine injection, except in Experiment 2, in which they were sacrificed 1 hour after the injection. The thyroid gland together with the trachea was dissected out. One lobe of thyroid was dissolved in 10 cc. 2 N NaOH while the other lobe, still attached to the trachea, was fixed in Bouin's fluid.

In Experiment 3 the animals were kept in metabolism cages so that urine and feces could be collected separately. The cages were moistened with 2 N NaOH to trap the iodine in the urine, care being taken so that the animals would not come in contact with the alkali. The radioactive content of these collections and of bladder, intestine, and carcass was investigated by dissolving each in a known amount of 2 N NaOH, plating 1 cc. of the dissolved organ, and counting the evaporated residue with a Geiger-Muller counter. The per cent of the injected dose present in an organ was calculated for the complete organ. Since a count was made only on one lobe of the thyroid, the counts obtained for this organ were multiplied by 2. The I^{131} in the samples has been expressed as percentage of the original I^{131} injected, by comparison with a dilution of the original solution measured under the same conditions as the sample.

To test the effectiveness of the stressing agent, the adrenals, thymus, and stomach of each animal were examined. In all cases of stress the adrenals were increased in size, the thymus decreased in size, while ulcerations of the stomach were noted in many of the rats. The weights of adrenals and thymus are recorded with the results of Experiment 4. The ratios of body weight to thyroid weight were not found to be significantly different in the various groups.

Coated radioautographs of thyroid sections of the animals in Experiments 1, 2 and 3 were made with Medium Lantern Slide Emulsion according to the method of Bélanger and Leblond (1946), as modified by Leblond, Percival and Gross (1948).

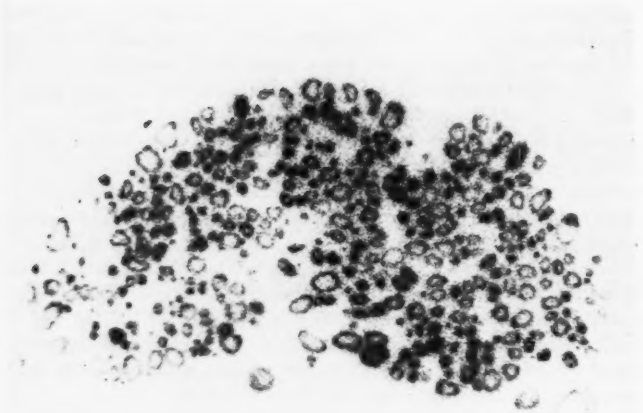
RESULTS

Experiment 1.—Per cent I^{131} in Thyroid 2 Hours after Subcutaneous Injection of I^{131}

Five groups consisting of 1) fed controls, 2) fasted controls, 3) forced muscular exercise, 4) spinal cord sectioned, and 5) formalin injected animals were sacrificed two hours after a subcutaneous injection of I^{131}. Groups 2 to 5 were fasted from the beginning of the experiment. The per cent of the injected dose taken up by the thyroid gland in each group during this interval is seen in Table 1, Column 1. There was no significant difference in the uptake of I^{131} between fed control animals and controls

TABLE 1

Treatment	Average % I^{131} uptake in thyroid gland 2 hours after subcutaneous injection of I^{131}	Average % I^{131} uptake in thyroid gland 1 hour after intravenous injection of I^{131}
Fed control	4.0±0.7	5.6±0.3
Fasted control	4.2±0.6	5.8±0.9
Fasted plus forced muscular exercise	3.2±0.9	3.1±0.8
Fasted plus formalin injection	0.8±0.2	1.1±0.1
Fasted plus spinal cord section	0.7±0.07	1.4±0.2



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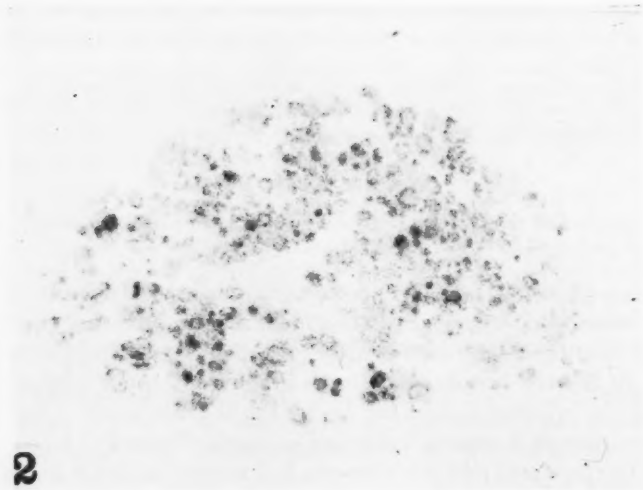

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The histological thyroid sections of both control and stressed animals were treated identically and at the same time. Since the coating with emulsion, the length of exposure, the development and fixation of the preparations and the photography were comparable for control and stressed rats, the intensity of the photographic blackening may be used as an index of the relative amount of radioactivity (Protein-bound-iodine) present in the thyroids of these animals.

FIG. 1. Unstained coated radioautograph of the thyroid gland of a control rat injected subcutaneously with I^{131} and sacrificed two hours later.

Most follicles appear as rings because of the deposition of photographic granules at the level of the epithelium.

FIG. 2. Unstained coated radioautograph of the thyroid gland of a rat which had been

which were not given food. However, there was a marked decrease in the uptake in the animals with spinal cord section and those injected with formalin, while there was only an insignificant decrease in the uptake in thyroids of animals given forced muscular exercise.

The intensity of the radioautographs agreed with the results that had been obtained chemically. In order to obtain the same density or amount of blackening of the photographic emulsion in stressed as in control animals, it was necessary to expose the autographic preparation of the thyroids from stressed animals for a much longer interval of time than was needed for preparations from unstressed animals.

The sites of distribution or localization of the radioactivity present in the glands of the stressed animals was not markedly different from the sites of radioiodine in control animals. (See Figs. 1 and 2.) In all groups most of the radioactivity was present in the cells of the follicles except for the central region of the gland which showed the radioactivity in the colloid of the follicles. It was expected that more cellular reactions would be found in the less active thyroids of the stressed animals. This was not the case. In fact, the trend was rather toward a greater number of cell reactions in the controls. This suggested that the thyroglobulin formed in the cells of stressed animals passed into the colloid at a rate at least equal to that in control animals.

A decrease in uptake without a decrease in the rate of passage from cell to colloid would suggest some inhibition of iodine entry without true decrease in thyroid activity. Several experiments were carried out to see whether this was really the case. In the second experiment, intravenous injection was used instead of subcutanous injection to make sure of the entry of radioiodine into the circulation.

Experiment 2.—Per Cent I^{131} in Thyroid 1 Hour after Intravenous Injection of I^{131}

Five groups of animals treated as above were sacrificed one hour after an intravenous injection of I^{131}. The results in Table 1, column 2 show the same general effect as was seen in Experiment 1, that is, severely stressed animals did not have as much radioiodine in their thyroids as did normals at this time interval. This difference was presumably not due to circulatory disturbances.

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stressed by formalin injections prior to a subcutaneous injection of I^{131}. The rat was sacrificed two hours after the I^{131} injection.

Although most follicles again show cellular reactions, there appears to be more colloid reactions in this radioautograph than in that seen in Fig. 1.

Compare the intensity of the radioautographic reactions with that in Fig. 1.

The radioautographic studies revealed that the intensity of the radioautographic reactions again was proportional to the counts contained in the glands. Again, there was no marked difference in the sites of activity, with perhaps more cellular reaction in controls. Since very little radioiodine was

TABLE 2

EXPERIMENT III

Percentage I^{131} Uptake in Different Organs in Stressed and Normal Rats Two Hours after a Subcutaneous Dose of I^{131}

	Thyroid	Urine and bladder	Feces and intestine	Carcass	Recovery
Fasted control	4.6±0.9	0.13±0.04	4.6±0.4	83.4±4.7	92.7
Fasted and formalin injection	0.6±0.07	7.00±1.68	3.2±0.6	55.0±3.9	65.8
	0.01>P>0.001	0.01>P>0.001	0.1>P>0.05	0.01>P>0.001	

entering the thyroid gland in the stressed rats, the fate of the injected radioiodine in the rest of the body was investigated in the following experiment.

Experiment 3.—I^{131} Distribution Studies 2 Hours after Subcutaneous Injection of I^{131}

The fate of the radioiodine was studied by determining the percentage uptake of I^{131} in the organs of excretion, the excreta, and the carcass, as well as in the thyroid gland. Since the injection route did not make any apparent difference and formalin injected animals gave consistent and representative results, control and formalin injected animals were sacrificed 2 hours after a subcutaneous injection of I^{131}. The results are seen in Table 2. The percentage uptake of I^{131} in the thyroid gland, as in the previous two experiments, showed a marked decrease in the uptake of I^{131} in stressed animals. There was also a decrease in the amount of I^{131} found in the carcass of these animals. The percentage uptake in the feces and intestine were not significantly different in the two groups. However, the urine and bladder of the stressed animals showed that far more radioiodine was excreted through the urinary system by stressed animals than by controls during this two hour interval. The total recovery of I^{131} in the animals of the formalin treated group (65%) was consistently less than that in the control group. The balance was presumed to have been lost in the urine which was so concentrated that a loss of one or several drops would result in a great loss in the amount of radioactivity. At any rate, this would reinforce the conclusion that much of the iodine in severely stressed animals is very rapidly lost through the urine as inorganic iodide.

The radioautographic results of this experiment were the same as in Experiment 1.

Experiment 4.—Per Cent I^{131} in Thyroid 2 Hours after a Subcutaneous Injection of I^{131} with Carrier Iodide

Control and formalin injected animals were sacrificed 2 hours after a subcutaneous injection of radioiodine mixed with 1 mg. of inorganic iodide. The results, as seen in Table 3, indicate that even if an excess of iodide is available to the glands, the thyroids of severely stressed animals

TABLE 3

EXPERIMENT IV

Percentage I^{131} Uptake in Thyroid 2 Hours after a Subcutaneous Injection of I^{131} Containing Carrier Iodine

	% I^{131} uptake in thyroid	Adrenal weights mgms.	Thymus weights mgms.
Fasted control	0.115 ± 0.011	18.2 ± 0.9	299 ± 25
Fasted and formalin injection	0.066 ± 0.018	28.6 ± 1.8	144 ± 17
"p"	0.05	0.001	0.001

can still only pick up little more than half of what the control thyroids are capable of capturing during this period.

DISCUSSION

During acute stress, the thyroid glands of animals consistently take up less radioiodine than do the glands of normal rats. The experiments showed clearly that there was a significant decrease in iodine uptake by the thyroid in the spinal cord sectioned group and in the formalin injected group, (the latter result in agreement with Paschkis *et al.*, 1950.[1] In the forced muscular exercise group, the decreased uptake was not significant in the first experiment but was so in the second. It is possible that there is in this case a situation similar to that arising in the case of cold in which there are two antagonistic effects produced on the thyroid: 1) specific to cold, which is to activate the gland, and 2) non-specific, which is due to stress, and decreased the activity of the gland. Thus, in the work of Leblond, Gross, Peacock and Evans (1944), the uptake of I^{131} was increased by cold but only until the resistance of the animals broke down. It is possible that

[1] Money *et al.* could not demonstrate a decrease in I^{131} uptake in thyroids of rats injected with 1 cc. 4% formalin daily for 10 days. This may have been due to the fact that the animals were already in the resistance phase or had adapted to the effect of the injections.

forced muscular exercise, like cold, may tend to raise the thyroid activity, possibly because this stimulus, like cold, influences basal metabolism. The concomitant stress would have an opposite effect which, in the second experiment, predominated and produced a significant decrease in the uptake of the radioiodine.

The metabolism of iodine in the thyroid gland may be subdivided into a number of phases: 1) the uptake of iodide by the so-called "iodine concentrating mechanism" (Raben and Astwood, 1949); 2) the synthesis of thyroglobulin in the cells; 3) the deposition of this thyroglobulin in the colloid; 4) the breakdown of thyroglobulin into its amino acid components; and 5) the passage of one of these, thyroxin, into the circulation (Leblond and Gross, 1948; Gross and Leblond, 1951). It is apparent that the I^{131} uptake of the thyroid may be modified by factors influencing any one of these steps so that the decrease produced by stress in the first experiment may be attributed to a modification of either one of several of the steps of thyroid activity. In the case of Experiment 4, however, the results estimate only the activity of the "iodine concentrating mechanism," since it dealt with the entry of large amounts of iodide into the gland (Leblond and Sue, 1941; Leblond, 1948). It may, therefore, be concluded that stress decreased the activity of the iodine concentrating mechanism.

It is known that this iodine concentrating mechanism is influenced by thyrotrophic hormone (Leblond and Sue, 1941) but whether or not in the case of stress its slowing down is due to a decrease in the level of this hormone can only be surmised. The results do not eliminate the possibility of a direct action on this mechanism by metabolites released during stress, such as cortical hormones.

The difference in the radioautographs between control and stressed animals suggests that stress has not only affected the iodine concentrating mechanism but somehow influenced steps 2 and 3 of thyroid function, since cellular reactions predominated in control while colloid reactions were more common in stressed animals. This discrepancy might be a consequence of the effect of stress in general on the peripheral metabolism of iodine. Thus, two hours after injection of radioiodine, a much larger percentage of it has been excreted into the urine of the stressed animals than in the controls. There would, therefore, be a loss of iodide at this stage, a fact also observed in man by Perry and Gemmel, 1949. These authors found that the urinary excretion of iodine was greatly increased during the first 24 hours after surgical operation in otherwise normal patients. This would suggest that a relative iodine deficiency occurs in the body during acute stress. After relative iodine deficiency it is our experience that cellular reactions are rare, while they are common when the level of circulating

iodide is high. Thus, the lack of cellular reactions in stressed animals would be due to the loss of the iodine stores from the body in stress.

From the data presented, it is clear that stressing agents which activate the adrenals cause 1) a loss of iodide ion into the urine and 2) a decrease in the activity of the "iodine concentrating mechanism" of the thyroid gland. The latter may be due to a decrease or suppression of thyrotrophic stimulation.

SUMMARY

The uptake of radioiodine by the thyroid glands of normal and severely stressed rats was investigated both chemically, using the Geiger counter method, and histologically, using the radioautographic technique.

Two hours after a subcutaneous or intravenous injection of I^{131}, the thyroids of animals subjected to either formalin injection or cervical cordotomy had taken up 6 to 8 times less radioiodine than had thyroids of normal animals. The uptake in the thyroids of animals submitted to forced muscular exercise was significantly decreased in only one of two experiments.

During this two hour interval, stressed animals excreted a large per cent of the injected radioiodine into the urine while control animals excreted relatively little. It appeared that in stressed animals radioiodine was not concentrated by the thyroid as much as in normal animals. On the other hand, in stress iodine was rapidly excreted into the urine.

To determine the activity of the "iodine concentrating mechanism" in the thyroid gland, the I^{131} uptake two hours after administration of radioiodine containing a large dose of carrier was measured. This was found to be decreased following stress. Since the activity of the "iodine concentrating mechansim" is known to be modified by thyrotrophic hormone, it was suggested that stress caused a decrease or suppression of thyrotrophic stimulation. A lowered thyrotrophic secretion would then cause a decreased uptake of iodine by the thyroid gland.

Acknowledgments

This work was supported by a grant from the National Research Council of Canada to Dr. C. P. Leblond. The junior author held a Canadian Life Insurance Officers Association Fellowship.

The authors wish to express their sincere appreciation to Dr. C. P. Leblond for continuous, stimulating direction during the experimental work, and helpful advice and active participation in the preparation of this manuscript.

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EXPERIMENTALLY INDUCED CHANGES IN THE MELANOPHORE HORMONE CONTENT OF THE RAT PITUITARY[1]

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OF THE manifold principles elaborated by the mammalian pituitary gland, the melanophore hormone (intermedin) remains one of the most puzzling, especially as regards its possible role in normal physiological processes. The amount of intermedin in the pituitary glands of a number of mammalian species compares favorably, per unit weight, with that found in amphibia. For example, the pituitary of the frog, *Rana pipiens*, contains approximately 500,000 units[2] per gram wet weight. Similar extraction procedures, applied to pools of glands, give values of about 400,000 units/gm. for the rat, 100,000 for sheep, and 600,000 for hog, while assays of individual mouse glands indicate levels ranging from 50,000 to 1,000,000 units/gm. Comparable data are also given by Zondek and Krohn (1932), using the *phoxinus* assay.

Waring and Landgrebe (1950) have recently reviewed the available evidence relating to the possible functions of intermedin in the mammal, and have concluded that reasonable basis exists for the implication of this hormone as a factor in melanin synthesis and as an antagonist of the hyperglycemic action of adrenalin. Evidence regarding other proposed physiological roles is less convincing.

The interpretation of observations of the effects of intermedin administration is complicated by uncertainties regarding the chemical and biological homogeneity of the preparations which have been employed for this purpose. An alternative approach to the problem of its physiological function has been employed in this investigation, based upon the assumption that interference with a normal physiological process in which intermedin is, directly or indirectly, involved should lead to significant changes in the intermedin content of the pituitary of the experimental animal. Detection

Received for publication June 9, 1951.

[1] This work was supported by a grant from the American Cancer Society to Harvard University. The technical assistance of Sylvia Green and Laurel Gans is gratefully acknowledged.

[2] The unit referred to is that defined by Frieden, Fishbein and Hisaw (1948), and is approximately equivalent to the melanophore hormone content of 0.1 μgm. of standard posterior lobe powder.

of such changes would be presumptive evidence for implication of the hormone, and provide a starting point for more detailed investigation.

This paper summarizes the results of experiments dealing with the effects of the administration of exogenous intermedin, as well as data which strongly support the possibility of a thyroid-melanophore hormone relation. Several reports have suggested this possibility; most directly related are the reports of blanching of amphibian larvae reared in solutions of thiouracil (Lynn and de Marie, 1946) and the observation by Giroud, Kfouri, and Martinet (1944) that thyroidectomy increases the intermedin content of the rat pituitary, whereas administration of thyroxin had the opposite effect.

METHODS

Rats of both sexes, from an inbred colony maintained at the Biological Laboratories were used. The animals were killed by a blow on the back of the neck. The hypophyses were removed, weighed, and stored in the deep-freeze in paraffined vials until assayed. For assay, each gland was twice extracted with 2 ml. of hot 0.25% acetic acid. The extract was diluted to 10 ml. and assayed immediately.

The application of the *in vitro* intermedin assay (Frieden, Fishbein and Hisaw, 1948) to the rat pituitary has been found to require certain departures from the usual technique. The standard dose-response curve for the assay was originally established for a preparation of intermedin made from sheep pituitary extracts. The use of this curve has been found to give consistent results, over a wide range of concentrations, for the assay of both hog pituitary extracts and standard posterior lobe powder (cattle). When applied to rat pituitary extracts, however, it was found that in addition to wide variation in response obtained upon repeated assay of the same sample, the slope of the dose-response curve seemed to be significantly different from that of the other species mentioned.

The procedure finally adopted was as follows. Six pituitaries from adult rats were pooled, ground, and extracted with dilute acetic acid. The extract was stored frozen in small aliquots. A total of about 50 assays of this extract, covering a wide range of concentration and response, was used to establish a dose-response curve. For subsequent assays of individual glands, each extract was assayed at at least four dilutions. The data (Δ_1/Δ_2, log D) for each gland were plotted and the best-fitting line of slope corresponding to that found for the pool extract was drawn. From this, the concentration of the original solution was calculated by assigning to the dilution corresponding to $\Delta_1/\Delta_2 = 0.5$ the value of 0.18 u/ml. (see Frieden, Fishbein and Hisaw, 1948). The results are expressed as units per milligram whole pituitary. In some instances, when the glands were not weighed, the pituitary weight was calculated from the body weight, using an average factor calculated for animals of similar ages.

For the experiments dealing with the effects of exogenous intermedin two preparations were employed. Both were prepared by ethanol fractionation of a hot dilute acetic acid extract of whole pituitary glands of hogs. Preparation P_I-XV-3 was about 3.0, and P_I-XVI-3 1.3 times as potent as standard posterior lobe powder.

RESULTS

Effect of Intermedin administration upon Intermedin content of the rat pituitary

Two sets of experiments were performed. In Table 1 are summarized the results of the first of these, in which the hormone levels of the glands

TABLE 1. EFFECTS OF INTERMEDIN ADMINISTRATION UPON THE MELANOPHORE HORMONE CONTENT OF RAT PITUITARY

Group	Number of animals	Age when killed (weeks)	Average body weight (gm.)	Treatment	Intermedin (units/mg.) ±S.E.
C	12	8	200	None	1640 ± 93
A	11	8	200	P_I-XV-3, 1.0 mg./day 14 days	1130 ± 148

TABLE 2. EFFECTS OF INTERMEDIN ADMINISTRATION UPON THE MELANOPHORE HORMONE CONTENT OF RAT PITUITARY

Group	Number of animals	Age when killed (weeks)	Average body weight (gm.)	Treatment	Intermedin (Units/mg.) ±S.E.
X	7	6	142	None	316 ± 100
O	6	14	230	None	410 ± 81
AA	7	16	335	None	540 ± 70
Q	6	18	307	None	540 ± 120
BA	9	36	355	None	440 ± 100
L	8	10	230	P_I-XV-3, 5.0 mg./day 15 days	70 ± 13
M	6	12	220	P_I-XVI-3, 5.0 mg./day 26 days	81 ± 10

of young male rats which had received 14 daily subcutaneous injections of 1 mg. P_I-XV-3 are compared with data for a similar group of untreated controls. Both groups were killed the day following the last injection. The difference in means of the two sets of data corresponds to a t value of 2.96, indicating a high level of significance ($p = <0.01$) for the difference.

The second set of experiments, summarized in Table 2, includes data for two groups of treated animals and five control groups of various ages. Group L received 5 mg. P_I-XVI-3 subcutaneously for 15 days, and were killed on the 16th day, while group M received the same treatment for a total of 26 days, and were killed on the 27th day. When compared with the data for control groups X and O (bracketing the ages of the treated animals), t values ranging from 2.14 ($p = 0.058$) to 4.80 ($p = 0.01$) are found. The data are thus of considerable significance.

The difference between the average values for the untreated series of

Table 1 and those of Table 2 has not been satisfactorily explained. All six groups were from the same colony, all were males, and the weights and ages were entirely comparable. The pituitaries of the control group of Table 1 were stored from 3–6 weeks in the deep-freeze, while the storage period for groups A, X, and BA were somewhat longer ($2\frac{1}{2}$–4 months). However, the data for most of group O and for group AA were obtained after storage periods of 1–7 weeks, and compare favorably with the others. More recently, assay of an extract of a total of ten pituitaries of twelve-week-old males within two days after collection gave an average value of 470 units/mg. Finally, only slight changes are observed in intermedin

TABLE 3. INTERMEDIN CONTENTS OF THE PITUITARIES OF HYPO- AND HYPERTHYROID RATS

Group	Number of animals	Average body wt.	Treatment	Intermedin (Units/mg. ±S.E.)
E	7	210	None	2690 ± 520
F	6	—	Thiouracil, 0.1%, 15–18 weeks	1380 ± 260
G	7	—	Thyroid powder, 0.2%, 4–5 weeks	3500 ± 520

TABLE 4. INTERMEDIN CONTENTS OF THE PITUITARIES OF THYROIDECTOMIZED AND THIOURACIL-TREATED RATS

Group	Number of animals	Age when killed (weeks)	Average body weight (gm.)	Treatment	Intermedin (Units/mg. ±S.E.)
J-I	8	12	150	Thyroidectomized, 5 weeks	39 ± 10
J-II	8	17	170	Thyroidectomized, 10 weeks	220 ± 39
J-III	7	25	175	Thyroidectomized, 18 weeks	280 ± 53
I	9	24	200	Thiouracil, 0.1%, 20 weeks	100 ± 22
K	6	56	255	Thiouracil, 0.1%, 20 weeks; untreated, 36 weeks	165 ± 41

content of hog pituitaries during many months of storage in the frozen state. Whatever the cause of the discrepancies noted, it is clear that the rat pituitary responds in a predictable manner to the administration of exogenous hormone.

Intermedin-thyroid relationship

Preliminary experiments on this phase of the problem were carried out using adult female rats treated with thyroid powder and thiouracil. As indicated in Table 3, decreases are observed in the intermedin content of the pituitaries of animals given thiouracil (groups E and F, $t = 2.16$, $p = 0.05$–0.10). Although increases are noted in hyperthyroid animals, the wide variation in response encountered renders this difference statistically doubtful.

The study of the effect of hypo- and hyperthyroid states was subsequently extended to male rats. The data in Table 4 include three groups of thyroidectomized rats, killed at intervals of 5, 10, and 18 weeks after thyroidectomy. Comparing each set of data with that for untreated animals of similar ages, it may be seen that the effect of thyroid removal is followed by a sharp decrease in the intermedin content of the pituitary. A trend toward restoration of normal values is noted during the next two months, a difference of borderline significance ($p = 0.05$–0.10) being observed $3\frac{1}{2}$ months after operation.

The effect of prolonged administration of thiouracil was studied in a

TABLE 5. INTERMEDIN CONTENTS OF THE PITUITARIES OF RATS FED DESICCATED THYROID POWDER (DTP)

Group	Number of animals	Age when killed (weeks)	Average body weight (gm.)	DTP in diet (%)	Length of treatment (weeks)	Intermedin (units/mg. ±S.E.)
R	6	11	175	0.2	3	970 ± 100
R′	8	12	155	0.2	4	300 ± 96
S	10	18	195	0.2	10	365 ± 89
T	7	21	225	0.2	13	640 ± 65
U	6	10	150	1.0	1.7	690 ± 160
V	9	10	155	1.0	2.1	175 ± 29
W	7	18	200	1.0	10	730 ± 220

group of 15 animals which received 0.1% thiouracil in drinking water during 5 months. Nine of the animals were then killed (group I); the others were maintained without the drug for another eight months (group K). The results obtained with group I substantiate the impressions derived from the preliminary experiments, i.e., a significant decrease (23% of the average for group BA, $p = 0.01$) occurs following administration of this antithyroid drug. The apparent diminution effected by thiouracil is somewhat exaggerated due to the pituitary hypertrophy which accompanies the development of a hypothyroid state (since the data are expressed in terms of units/mg. gland weight). However, when the average total amounts per gland are compared, group I is still only 36% of group BA.

Restoration of normal intermedin levels following withdrawal of thiouracil seems to be very slow, as indicated by the data for group K. It is of interest that when these were killed marked thyroid hypertrophy was observed in all but two of the animals, and these two were found to have the highest intermedin content.

The effects of administration of thyroid powder at two levels is indicated in Table 5. Parke, Davis desiccated thyroid powder ("strong") was mixed with powdered Purina feed in the proportions indicated. This was fed *ad lib.* At both levels a short-lived increase in melanophore hormone was

observed shortly after the regimen was instituted. The difference for group R ($p = 0.01$, compared with control group O) is of considerable significance; that for group U ($p = 0.15$, compared with group O) is much less certain. Prolonging the thyroid treatment results in an abrupt fall, followed by a slow increase in intermedin levels to values somewhat above the normal. The trend of the data suggests that more striking effects might be observed if the effects of shorter periods of thyroid hormone administration were examined. Such studies are currently in progress.

DISCUSSION

Two general conclusions may be drawn from the experiments set forth here. It is clear that the concentration of intermedin in the pituitary is influenced by the level of the hormone in the body. This has previously been observed only in the frog (Frieden and Bozer, 1951). This is a further indication of the probable importance of intermedin in mammalian physiology.

The second observation of significance concerns the establishment of a relation between the melanophore hormone and hyper- and hypothyroid states. The results obtained here are in clear contradiction to those reported by Giroud, Kfouri, and Martinet (1944). Their data, based upon assays using isolated carp scales, were confined to a very few animals; in view of the wide variability encountered between values for individual rats, no real importance can be attached to the small differences noted. On the other hand, the effects of thiouracil upon intermedin physiology in the rat parallels that observed by Lynn and de Marie (1946) in the tadpole.

It is of interest to note that the rat can apparently adapt, with respect to pituitary intermedin, to a permanently hypothyroid condition induced by thyroidectomy, whereas continued treatment with thiouracil results in apparently permanently reduced intermedin levels. It is also worthy of reiteration that the effect of thyroid administration, which is opposite in sense to the effect of thyroidectomy, is likewise transitory.

The question of the specific function of the melanophore hormone in mammals remains an open one, but speculation concerning it has now a sounder basis. In addition to a possible role in processes in which the thyroid is also involved, intermedin has been shown (Frieden and Bozer, 1951) to be concerned in the synthesis of melanin in the frog. The common meeting-ground would appear to be metabolic phenomena centering about tyrosine and its oxidation products. The observation of Koneff, Simpson, and Evans (1946) that prolonged treatment of hamsters with stilbestrol results in pronounced hypertrophy of the pars intermedia suggests that the metabolism of other phenols may also be involved.

Some years ago, Fostvedt (1939, 1940) reported the enhancement of tyrosinase activity by intermedin-containing preparations, using tyrosine as a substrate, but the effects noted could not definitely be ascribed to the hormone. We have repeated some of these experiments using tyrosinase preparations derived from mushrooms; although positive results have sometimes been obtained, they have not so far proved reproducible. It would seem more desirable, however, to use for this purpose tyrosinase preparations from mammalian sources, e.g., tyrosinase from melanoma tissue.

Acknowledgment

It is a pleasure to acknowledge the advice and encouragement received from Dr. F. L Hisaw during the course of this study.

SUMMARY

Daily administration of intermedin preparations (from hog pituitary glands) to rats for periods of two to four weeks results in decreases of 30–80% in the average melanophore hormone content of the pituitary. Decreases of even greater magnitude are observed following both thyroidectomy and the administration of thiouracil. Melanophore hormone levels of the pituitaries of thyroidectomized animals are gradually restored during periods of one to three months, while the effects of thiouracil persist both during treatment and for a considerable period following withdrawal of the drug. Slight transitory increases in melanophore hormone content were observed in animals made hyperthyroid by feeding desiccated thyroid powder.

These observations further suggest the probable importance of intermedin in mammalian physiology.

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